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## Introduction:

Beta-catenin plays a key role in the Wnt signaling pathway. When Wnt protein binds to its receptor, frizzled, downstream signaling events result in the accumulation of beta-catenin in the cytoplasm and nucleus where it interacts with TCF/LEF transcription factors. The Wnt/beta-catenin and tumor necrosis factor alpha (TNF $\alpha$ )/IkappaB/NFkappaB pathways are involved in cell cycle control, differentiation and inflammation. Both IkappaB and beta-catenin are regulated by phosphorylation at similar consensus serines (within consensus sequence, DSGIHS) at the N-terminal and are targeted for ubiquitination and degradation by the same ubiquitin ligase complex. The IkappaB kinase (IKK) complex that phosphorylated IkappaB contains 2 kinases, IKK $\alpha$  and IKK $\beta$ . It has previously been shown that the IKK complex also interacts with and phosphorylated beta-catenin and can regulate its dependent transcriptional activity. Several studies have shown that Wnt and NFkappaB pathways interact, where Wnt-1 can activate NFkappaB through inhibition of GSK-3 $\beta$ , although it is not clear how this occurs. Evidence was also shown that total beta-catenin was localized within the nucleus of IKK $\alpha$  -/- murine cells, which indicate a possible role for beta-catenin in regulating epithelial cell proliferation and differentiation. We wanted to determine how a well studied cytokine, like TNF $\alpha$ , which has been shown to dramatically increase NFkappaB signaling activity may regulate beta-catenin transcriptional activity and if the increase in NFkappaB activity might be involved. Furthermore, cytokines like TNF $\alpha$  have been shown to play a role in epidermal morphogenesis. Ectodysplasin, and its receptor, Edar are recent additions to the TNF family. Since the phenotype of IKK $\alpha$  -/- mice and the mice counterpart of Edar, *dl* are similar, we wanted to determine if ectodysplasin may also affect beta-catenin and be involved in regulating epidermal development. We show in this study through the use of various approaches that IKK $\alpha$  and IKK $\beta$  but not NFkappaB mediate the effects of TNF $\alpha$  on beta-catenin signaling activity. By understanding the different means of regulating beta-catenin transcriptional activity, we may begin to understand and control cancer development.

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Body:

**1. To determine the effect of TNF $\alpha$  on  $\beta$ -catenin signaling activity in SW480 cells.**

Initial experiments to determine if TNF $\alpha$  had any effect on  $\beta$ -catenin signaling activity were carried out by treating SW480 colon cancer cells and HEK 293 cells with 20ng/ml TNF $\alpha$  for sixteen hours. SW480 cells have a truncated APC gene and are unable to effectively degrade  $\beta$ -catenin, leading to high endogenous levels of  $\beta$ -catenin signaling. HEK 293 cells do not have high endogenous levels of  $\beta$ -catenin and are useful to rule out the effects that the high  $\beta$ -catenin levels in SW480 cells may have on the observations. To measure  $\beta$ -catenin signaling, a luciferase promoter (Topflash) with four upstream TCF/LEF response elements was used, which allow binding of TCF/LEF transcription factors. The amount of light produced is measured in lumens. A TCF/LEF mutated luciferase reporter (Fopflash) was used as a negative control. The NF $\kappa$ B luciferase reporter has several NF $\kappa$ B binding sites upstream of a thymidine kinase promoter. NF $\kappa$ B reporter was used as a positive control since it is known that it is activated by TNF $\alpha$  (1). Renilla luciferase reporter was used as an internal control to correct for transfection efficiency. All transfections were done in triplicates and repeated at least 3 times after which statistical analysis was performed on the data. In this experiment, SW480 cells were transfected with either Topflash, NF $\kappa$ B or Fopflash reporter for twenty-four hours and treated with TNF $\alpha$  for sixteen hours. The results show that TNF $\alpha$  decreased  $\beta$ -catenin signaling activity by 63% and increased NF $\kappa$ B signaling activity by 187% (**Figure 1.1A-B**) Since Fopflash reporters have mutated TCF sites and are not able to bind to  $\beta$ -catenin, little signaling was observed. These initial results show that TNF $\alpha$  repress  $\beta$ -catenin signaling activity.

**2. To determine if over-expression of ectodysplasin receptor, Edar has any effect on  $\beta$ -catenin signaling activity.**

The ectodysplasin receptor, Edar was overexpressed by transfecting SW480 colon cancer cells with 50ng Edar and either 0.1ug of Topflash, Fopflash or NF $\kappa$ B reporters. Edar decreased  $\beta$ -catenin signaling activity by 87% and significantly increased the NF $\kappa$ B signaling activity by about 200% (**Figure 1.2A**). Similar experiments were performed with the ligand, Eda-A1 and found that it was also able to slightly decrease  $\beta$ -catenin signaling by 26%. This was somewhat surprising since the expression of the Eda receptor, Edar has previously been reported only in the skin (**Figure 1.2B**). It is expected that the overexpression of Edar would result in a larger repression compared to the overexpression of the ligand itself since the number of receptors per cell may be a limiting factor and addition of the ligand alone would result in a smaller degree of activation. Similarly, the increase in NF $\kappa$ B activity only increased by 133%. In the presence of Edar, overexpression of the ligand results in a constitutively active system that stimulates downstream proteins after Eda-A1 is able to bind to its receptor, Edar. Because Eda-A1 was able to repress  $\beta$ -catenin signaling in SW480 cells, SW480 cells were examined to see if they expressed Edar. Similar experiments had been repeated in HEK 293 cells and so we also harvested RNA from these cells to determine if they had endogenous expression of Edar. A forward primer from position 1129 and a reverse primer from position 1725 was used and performed 1-step RT-PCR. A band of 597 bp in size was expected, which corresponds to Edar. Our results confirmed that SW480 cells and to a much lesser extent, HEK293 cells do express the receptor (**Figure 1.3**).

**3. To determine localization of  $\beta$ -catenin in IKK $\alpha$  (-/-) mice.**

A number of studies indicate that IKK $\alpha$  does not play a dominant role in the regulation of NF $\kappa$ B signaling [2-3]. Although the IKK $\alpha$  (-/-) mouse does not have a marked NF $\kappa$ B phenotype, it does exhibit a thickening of the epidermis and has a defect in keratinocyte differentiation [4-5]. Moreover, Hu et. al. had shown that the role of IKK $\alpha$  in keratinocyte differentiation is not exerted through IKK activation of NF $\kappa$ B [6]. Because elevated  $\beta$ -catenin signaling is associated with hyper-proliferation of epidermal basal cells, and the Wnt/ $\beta$ -catenin/TCF pathway is implicated in epithelial stem cell proliferation and differentiation,  $\beta$ -catenin localization in the epidermis of IKK $\alpha$  (-/-) mice was investigated. Unfortunately, the de-phosphorylated  $\beta$ -catenin antibody did not work on paraffin sections. For these experiments, a  $\beta$ -catenin antibody that detected the C-terminal of  $\beta$ -catenin protein was used. Membrane  $\beta$ -catenin staining was observed in both wildtype and IKK $\alpha$  (-/-) epidermis but a marked increase in the number of cells with nuclear  $\beta$ -catenin occurred throughout the epidermis and particularly in the basal region of IKK $\alpha$  (-/-) mice. (**Figure 1.4A-D**) Very few cells in the normal epidermis exhibited nuclear  $\beta$ -catenin staining at this stage in development. Nuclear  $\beta$ -catenin staining also occurs in human epidermal keratinocytes expressing N-terminal deleted form of  $\beta$ -catenin but not in normal keratinocytes or in normal human skin [7]. Localization of  $\beta$ -catenin in the nucleus suggests an increase in  $\beta$ -catenin/TCF signaling. Thus  $\beta$ -catenin target gene cyclin D1 was localized. Like  $\beta$ -catenin, cyclin D1 was markedly upregulated in the basal regions of the IKK $\alpha$  (-/-) mouse epidermis. (**Figure 1.4E-H**) This indicates that  $\beta$ -catenin and IKK $\alpha$  may have some interaction.

#### **4. Effect of constitutively active IKK mutants on $\beta$ -catenin signaling activity.**

Previous sections have shown that TNF $\alpha$  and Eda/Edar repress  $\beta$ -catenin signaling. Further evidence has also been shown that  $\beta$ -catenin nuclear staining is up-regulated in IKK $\alpha$  (-/-) fibroblast cells. This leads to examine if the individual IKK subunits, IKK $\alpha$  and IKK $\beta$  are involved in TNF $\alpha$  and Eda/Edar repression  $\beta$ -catenin signaling activity. To determine whether TNF $\alpha$  could regulate  $\beta$ -catenin signaling through the IKK complex, SW480 colon cancer cells were transfected with constitutively active IKK mutants. These constitutively active mutants (CA IKK $\alpha$  and CA IKK $\beta$  were generated by changing serine residues within the conserved region of the activation loop at position 176 and 180 in IKK $\alpha$  and 177 and 181 within IKK $\beta$  to glutamate) (SS to EE). This mutation results in a conformational change that is similar to its activated state when it is phosphorylated. SW480 cells were a good model to use because it has a truncated APC gene which prevents  $\beta$ -catenin from being degraded, resulting in a high level of endogenous  $\beta$ -catenin in the cell. As described above, reporters, Topflash, Fopflash or NF $\kappa$ B were used to measure  $\beta$ -catenin signaling activity. Again, experiments were done in triplicate and each experiment was repeated at least three times. TNF $\alpha$  reduced  $\beta$ -catenin signaling as shown before and CA IKK $\alpha$  and CA IKK $\beta$  by themselves also decreased  $\beta$ -catenin signaling by 82% and 90% respectively. As expected, the presence of both TNF $\alpha$  and CA IKK mutants further decreased its signaling by 5% for IKK $\alpha$  and 3% for IKK $\beta$ . It also further augmented NF $\kappa$ B signaling activity after treatment with TNF $\alpha$  by more than 2 fold confirming its role in activation of NF $\kappa$ B signaling (**Figure 1.5**). This suggests that IKK may have role in regulating  $\beta$ -catenin activity. Since CA IKK mutants decrease

Topflash activity, the next logical step was to determine if dominant negative IKK mutants could reverse this effect.

### **5. Effect of dominant negative IKK mutants on $\beta$ -catenin signaling activity.**

To further determine the contribution of the IKK complex in downstream events following TNF $\alpha$  stimulation, dominant negative IKK mutants (doses ranging from 0.1ug to 1.0ug) were transfected into the same cell lines together with Topflash, Fopflash or NF $\kappa$ B reporters. Experiments using cyclin D1-luciferase reporter in SW480 cells were repeated to show that observations are dependent on TCF binding elements, which are downstream targets of  $\beta$ -catenin. This cyclin D1 promoter construct (-163CD1Luc) has TCF/LEF sites as well as CREB, EGR1, SP1, E2F1 and NF $\kappa$ B binding sites (8-9). The dominant negative mutants (DN IKK $\alpha$  and DN IKK $\beta$ ) were generated by changing serine residues at position 176 and 180 to alanine in IKK $\alpha$  and 177 and 181 to alanine in IKK $\beta$  (SS to AA). This mutation prevents kinase activation in response to TNF $\alpha$  stimulation. Both DN IKK $\alpha$  and DN IKK $\beta$  were able to reverse TNF $\alpha$  down-regulation of  $\beta$ -catenin signaling in a dose-dependent manner (**Figure 1.6A**). Both dominant negative mutants were also able to block any increase in NF $\kappa$ B signaling activity after treatment with TNF $\alpha$ . DN IKK $\alpha$  was able to further increase Topflash signaling activity at its highest dose, unlike DN IKK $\beta$ . This may be a result of over-expressing large amounts of exogenous DNA in the cell system resulting in an over-stimulation effect. SW480 cells, as mentioned earlier, are unable to degrade  $\beta$ -catenin as a result of a mutated APC gene. Thus APC was added back to determine if this had any effect on the observations (**Figure 1.6B**). As expected, APC decreased  $\beta$ -catenin signaling by 83%, however, neither of the dominant negative forms of IKK were able to reverse the inhibition of  $\beta$ -catenin

signaling activity. In addition, APC did not potentiate or inhibit the ability of TNF $\alpha$  to regulate NF $\kappa$ B activity in SW480 cells. These data strongly suggest that the effects of IKK are independent of APC. These experiments were repeated in 293 cells, which have less endogenous  $\beta$ -catenin than SW480 cells as a result of an intact APC gene, with similar results (**Figure 1.6C**). This strongly indicates that both IKK $\alpha$  and IKK $\beta$  are involved in cytokine regulation of  $\beta$ -catenin signaling but are not required to mediate APC effects.

#### **6. Effect of silencing either IKK $\alpha$ or IKK $\beta$ when treated with TNF $\alpha$ .**

Over-expression of IKK through transfection of exogenous plasmids, although the easiest and the fastest way to determine its contribution in the pathway, is not the best method. The addition of large amounts of DNA may affect the stoichiometry of the IKK complex within the cell and this may cause unknown effects within the signaling pathway, which lead to misleading observations. Gene silencing technique was used to determine the importance of IKK in both TNF $\alpha$  and Edar down-regulation of  $\beta$ -catenin signaling activity. In gene silencing, double-stranded RNA (dsRNA) corresponding to IKK $\alpha$  or IKK $\beta$  is added to cells and rapid degradation of mRNA silences its expression within the cell. Appropriate dose was determined to silence each of the IKKs in SW480 cells through western analysis (**Figure 1.7A**) and treated cells with RNA oligonucleotides against IKK $\alpha$  and IKK $\beta$ . Repression of  $\beta$ -catenin signaling by TNF $\alpha$  was markedly inhibited in the presence of IKK $\alpha$  or IKK $\beta$  RNAi (**Figure 1.7B-C**). Both IKK $\alpha$  and IKK $\beta$  were further examined to determine their involvement in this pathway. We know that RNA interference has its shortcomings, since it is transient and there is increasing evidence that there may be non-specific inhibition and toxicity to cells. E.g. IKK $\beta$  RNA

at low doses, did not silence IKK $\beta$  in SW480 cells. The use of the RNAi at doses higher than 100nM resulted in cell death. Targets were also chosen at sites other than the current RNAi for IKK $\beta$ , however, it was also unsuccessful in knockout out specific gene expression. Since there was much technical difficulties in manipulation of the systems to prevent cell toxicity at high doses of RNAi, IKK $\alpha$  (-/-), IKK $\beta$  (-/-) and double knockout mouse embryonic fibroblasts (MEFs) were used to show that IKK is involved in TNF $\alpha$  and Eda/Edar regulation of  $\beta$ -catenin signaling. MEF cells were treated with TNF $\alpha$  for 16 hours after transfecting them with Topflash or NF $\kappa$ B reporters as described previously, and compared them with normal parental MEF cells. Both the IKK $\alpha$  (-/-) and IKK $\beta$  (-/-) cell lines were insensitive to TNF $\alpha$  repression of  $\beta$ -catenin activity when compared to the wild type. (**Figure 1.8**) NF $\kappa$ B activity in TNF $\alpha$  treated IKK $\alpha$  (-/-) and IKK $\beta$  (-/-) cell lines was reduced compared to wild type cells. This confirms that the IKK complex plays a regulatory role in TNF $\alpha$  down-regulation of  $\beta$ -catenin signaling activity. To further confirm the role of IKK, wild type IKK $\alpha$  and IKK $\beta$  were over-expressed in the knockout mouse embryonic cells and treated them with TNF $\alpha$ . As expected, addition of either of the IKKs restored TNF $\alpha$ -mediated repression of Topflash reporter activity and that addition of both IKKs to the double knockout cells decreased TNF $\alpha$  repression of  $\beta$ -catenin signaling almost 10 fold. (**Figure 1.9A-C**) Addition of both IKKs also augmented NF $\kappa$ B activity almost 2 fold. (**Figure 1.9D-F**)

Experiments were repeated by over-expressing Edar. (**Figure 1.10A-F**) Contrary to earlier results with dominant negative IKK mutants, the knockout MEF cells showed a similar repression in signaling activity even when either IKK $\alpha$  or IKK $\beta$  were absent.

(Figure 1.11) However, when both IKK $\alpha$  and IKK $\beta$  were removed in the double knockout MEFs, significant absence of TNF $\alpha$  repression was observed. (Figure 1.9) As explained earlier, the use of over-expression studies results in over-stimulation of the cell system. RNAi data were not perfect since there were difficulties in silencing IKK $\beta$  without resulting in cell toxicity. MEF cells, on the other hand are the best system to use since the gene is already absent. Thus taking into account these data, there is a strong indication that both IKK $\alpha$  and IKK $\beta$  are required to transduce the effects of Edar on  $\beta$ -catenin signaling activity and further confirms that both IKK $\alpha$  and IKK $\beta$  are required to mediate Edar regulation of  $\beta$ -catenin signaling. Recent evidence have shown that a new adapter protein, Edar associated death domain, (Edaradd), is involved in transducing signals from Edar. It was identified through the mouse crinkled locus. It is possible that over-expression of Edar results in signals transduced through Edaradd to the IKK complex which affects downstream signal pathways that affect  $\beta$ -catenin signaling activity. Also, the use of recombinant Eda would be most ideal to study its effects on  $\beta$ -catenin signaling in the cell. Immunoprecipitation of Edaradd would be an initial step to probe for proteins that might be in complex with it, such as IKK. On the other hand, TNF receptor, TNFR may be able to recruit heterodimers of IKK $\alpha$  and IKK $\beta$  thus when either one of the kinase is absent, homodimers are unable to transducer signals downstream of the receptor.

Thus we show that IKK $\alpha$  and IKK $\beta$  are both shown to be involved in repressing  $\beta$ -catenin signaling activity after stimulation by TNF $\alpha$ . This discovery shows that there is a novel pathway of regulation of  $\beta$ -catenin signaling by cytokines. Not much is known about this pathway and further studies are required to determine how this repression

occurs and whether  $\beta$ -catenin is degraded, providing an explanation for the decrease in signaling activity.

### **7. Influence of NF $\kappa$ B activity on TNF $\alpha$ / Edar repression of $\beta$ -catenin Signaling.**

As discussed above, phosphorylation of the consensus sequence, DSGXXS, found in both  $\beta$ -catenin and I $\kappa$ B stimulates their interactions with the ubiquitin ligase  $\beta$ -TrCP leading to their degradation via the ubiquitin-proteasome pathway [10-12]. One study has shown that the  $\beta$ -catenin/TCF complex increases  $\beta$ -TrCP levels by a post-transcriptional mechanism to result in a decrease of  $\beta$ -catenin and increase in NF $\kappa$ B activity [13]. In addition, disruption of either murine GSK-3 $\beta$  and IKK $\beta$  genes result in embryonic lethality due to hepatic apoptosis from increased sensitivity to TNF $\alpha$  [14]. These results suggest potential relationships between  $\beta$ -catenin and NF $\kappa$ B signaling pathways. Activation of NF $\kappa$ B signaling was examined to determine if it may indirectly be responsible for the cytokine repression of  $\beta$ -catenin activity. 80nM of RNA oligonucleotides against p65 subunit of NF $\kappa$ B were transfected into SW480 cells to silence the transcriptionally active form of NF $\kappa$ B i.e. p65. Confirmation that the gene was silenced was determined through western analysis of protein lysates after transfection and by luciferase assays using the NF $\kappa$ B reporter. In (**Figure 1.12A-C**), neither TNF $\alpha$  nor Edar activated NF $\kappa$ B signaling activity in the presence of p65 RNAi (**Figure 1.12D**). Despite the absence of NF $\kappa$ B,  $\beta$ -catenin signaling was still repressed by 34% after treatment with 20ng/ml of TNF $\alpha$ , while over-expression of Edar inhibited  $\beta$ -catenin signaling by 51%, showing that NF $\kappa$ B does not play a role in TNF $\alpha$  or Edar repression of  $\beta$ -catenin signaling (**Figure 1.12B, D**). This indicates that cytokine repression of  $\beta$ -

catenin activation is independent of the activation of NF $\kappa$ B signaling and that in our system, these 2 pathways are independent of each other. It is very likely that the repression of  $\beta$ -catenin signaling is a result of an increase in degradation of the protein.

### **8. Involvement of $\beta$ -TrCP in Down-regulating $\beta$ -catenin Signaling**

Previous data has ruled out a role for NF $\kappa$ B activation on the repression of  $\beta$ -catenin signaling activity. Attention was next focused on the possibility of increased degradation of  $\beta$ -catenin. Thus the possibility that an increase in  $\beta$ -catenin degradation as a result of its phosphorylation by the IKK complex was examined. Since it is well-known that  $\beta$ -TrCP is the F-box protein involved in the SCF ligase complex responsible for the degradation of  $\beta$ -catenin in the canonical Wnt pathway, an investigation was done to determine if this protein was involved in the TNF $\alpha$  and/ or Edar effects. Specifically, the hypothesis that TNF $\alpha$  and Edar inhibit  $\beta$ -catenin signaling through increased degradation of the protein as a result of targeted phosphorylation of  $\beta$ -catenin and recruitment of  $\beta$ -TrCP and subsequent ubiquitination and proteosomal degradation was tested. Luciferase assays and similar transfections on HEK 293 cells were performed. HEK293 cells have lower endogenous  $\beta$ -catenin and they were transfected with either dominant negative mutant  $\beta$ -TrCP1 or  $\beta$ -TrCP2 (DN  $\beta$ -TrCP) and treated with 20ng/ml of TNF $\alpha$  or transfected with 50ng of Edar (**Figure 1.13A, 1.14A**). As expected, when dominant negative mutant  $\beta$ -TrCP1 was present alone, there was a 23 fold increase in  $\beta$ -catenin signaling activity since  $\beta$ -catenin degradation is blocked. However, even with this marked increase in signaling activity, DN  $\beta$ -TrCP1 did not completely reverse TNF $\alpha$  or Edar repression of  $\beta$ -catenin signaling. There was still a 31% decrease in  $\beta$ -catenin

signaling when TNF $\alpha$  was added and a 74% decrease when Edar was overexpressed as compared to their controls. Thus  $\beta$ -catenin signaling was still significantly repressed when either TNF $\alpha$  or Edar was present, indicating that  $\beta$ -TrCP-mediated ubiquitination is only partly responsible for the inhibition in  $\beta$ -catenin signaling. Similar experiments with their controls were performed using NF $\kappa$ B reporter showing no significant induction of luciferase activity after treatment with TNF $\alpha$  or overexpression of Edar in the presence of DN  $\beta$ -TrCP1 (**Figure 1.13B, 1.14B**). This indicates that the DN mutant is blocking cytokine activation of NF $\kappa$ B activity as one would expect. It is surprising to observe that DN  $\beta$ -TrCP2 does not prevent NF $\kappa$ B activation after the addition of TNF $\alpha$  or Edar. It is possible that DN  $\beta$ -TrCP2 does not work as well as the dominant negative  $\beta$ -TrCP1. It is also possible that the cells do not express this F-box protein and thus are insensitive to the addition of a dominant negative mutant. Since antibodies are not commercially available, 1-step RT-PCR was utilized to determine the expression of the gene in the cell lines that the experiments were performed in, i.e. SW480 and HEK293 cells. Both of these F-box proteins are expressed in both cell lines ruling out the second explanation (**Figure 1.15A**). A schematic of the location of the primers used to detect the gene is shown in **Figure 1.15B**. Furthermore, the role of  $\beta$ -TrCP2 is not clear. It is interesting to note that even though  $\beta$ -TrCP1 and  $\beta$ -TrCP2 share 78% amino acid sequence homology, both are found on different chromosomes;  $\beta$ -TrCP1 is found on 10q24-25 and  $\beta$ -TrCP2 is found on 5q33-34 [15]. It is possible that even though both isoforms may share similar roles in ubiquitinating  $I\kappa B\alpha$ ,  $\beta$ -TrCP2 may have additional functions within the cell that involve other pathways. However, the data here indicates that  $\beta$ -TrCP1 may be involved in

degrading  $\beta$ -catenin protein after stimulation with TNF $\alpha$  or Edar but by itself, is not responsible for the decrease in signaling observed. This strongly indicates that a relocalization of  $\beta$ -catenin must occur, most likely from the nucleus to the cytoplasm or membrane.

### **9. TNF $\alpha$ / Edar Decreases Nuclear De-phosphorylated $\beta$ -catenin**

Previous sections have already ruled out NF $\kappa$ B activation in the repression of  $\beta$ -catenin signaling activity and degradation via the F-box protein,  $\beta$ -TrCP that is also involved in  $\beta$ -catenin degradation within the canonical Wnt pathway. Re-localization of  $\beta$ -catenin out of the nucleus is another possible explanation of the inhibition in its signaling activity. Earlier data generated from the Byers lab have shown that phosphorylation of certain serine residues in the N-terminal of  $\beta$ -catenin can regulate its stability by targeting it for ubiquitination [16]. Although a putative IKK consensus sequence is present in the N-terminal region of  $\beta$ -catenin and is a likely target for IKK, mapping studies indicated that several other regions of  $\beta$ -catenin can serve as substrates for IKK [16]. It is also possible that IKK may affect  $\beta$ -catenin mediated trans-activation by phosphorylating another component of the transcriptional machinery. If TNF $\alpha$  and Edar or activation of IKK regulates  $\beta$ -catenin signaling by targeting it for ubiquitination and protein degradation, one would expect that like APC, axin or activated GSK-3 $\beta$ , they would affect  $\beta$ -catenin protein levels [17-18]. If TNF $\alpha$ /IKK and Edar phosphorylation of  $\beta$ -catenin does not alter its degradation, it was hypothesized that it might alter the localization of the de-phosphorylated “active” form of  $\beta$ -catenin. Immunocytochemistry was used to test this hypothesis. Double-labeling studies of  $\beta$ -catenin in SW480 cells transiently transfected

with CA IKKs revealed no significant alteration in total  $\beta$ -catenin protein or localization in the cells expressing IKKs when compared to control cells. (**Figure 1.16A-C**) Recent papers by Clevers et al. have shown that level of an ‘active’ form of  $\beta$ -catenin is increased following Wnt stimulation [19]. Since there were no changes in  $\beta$ -catenin protein localization when probed with the C-terminus  $\beta$ -catenin antibody, an investigation was done to confirm if the changes in signaling activity may be due to changes in distribution of this ‘active’ fraction of  $\beta$ -catenin. CA IKK mutants were transfected into SW480 cells or treated with either TNF $\alpha$  or Edar and the ‘de-phosphorylated’  $\beta$ -catenin,  $\alpha$ -ABC antibody was used to probe for this ‘active’ fraction.

Immunocytochemistry shows that there is a marked decrease in nuclear staining after treatment with TNF $\alpha$  (**Figure 1.17A-B**) Similar results were obtained with the transfection of Edar (**Figure 1.18A-C**) or the overexpression of CA IKK mutants (**Figure 1.19A-C**). Data was quantitated and the number of cells with altered localization of phosphorylated  $\beta$ -catenin was determined by scoring 10 fields of view (**Figure 1.17C**). Thus no nuclear staining was observed with cells expressing Edar or CA IKK $\alpha$  or CA IKK $\beta$  indicating that either the de-phosphorylated form of  $\beta$ -catenin is degraded or relocalized out into the cytoplasm to be degraded. Note that this quantitation does not take into account cells with reduced but still detectable nuclear localization of activated  $\beta$ -catenin after treatment with TNF $\alpha$  and therefore represent an underestimate of the effects of TNF $\alpha$ . There was a significant number of cells which did not have any nuclear staining of the de-phosphorylated  $\beta$ -catenin. This ‘activated’  $\beta$ -catenin was almost always absent from the nuclei of cells transiently transfected with CA IKK  $\alpha$  or CA

IKK $\beta$ . (**Figure 1.19A-C**) In cells with CA IKK $\alpha$  expressed, only 12% of cells had nuclear staining and in cells with CA IKK $\beta$  expressed, only 6% of cells had nuclear staining. Not all cells have similar amounts of endogenous  $\beta$ -catenin present and the presence of CA IKK mutants may only be able to decrease a certain amount of  $\beta$ -catenin within the cell. Cells with more endogenous  $\beta$ -catenin may have 'residue' levels left which are detected by the antibody. These results confirm that the effects of TNF $\alpha$  and Edar on  $\beta$ -catenin signaling are not associated with degradation of total  $\beta$ -catenin protein but do involve changes in the localization of the 'activated'  $\beta$ -catenin fraction. Furthermore, this also confirms that there is a pool of  $\beta$ -catenin that is regulated by TNF $\alpha$  and Edar within the nucleus and this pool is responsible for the decrease in reporter activity. The results here suggest that the N-terminus of  $\beta$ -catenin is regulated by activated IKKs through phosphorylation of specific residues. In the next experiments, western analysis of the de-phosphorylated  $\beta$ -catenin antibody was used to detect any changes in protein expression of this 'active' fraction within the cell.

#### **10. TNF $\alpha$ repression of $\beta$ -catenin activity involves phosphorylation of N-terminal serine and threonine residues:**

Immunocytochemistry results indicate that TNF $\alpha$  and Edar might be regulating  $\beta$ -catenin at certain residues on the N-terminus. If so, then deletion constructs of  $\beta$ -catenin which do not have the amino terminus should be resistant to the effect of TNF $\alpha$  on  $\beta$ -catenin signaling activity. Deletion constructs were made by removing the N-terminus (1-142aa) or the C-terminus (623-781aa) of  $\beta$ -catenin and transfecting these constructs into wild type MEF cells (**Figure 1.20A**). Removal of the amino and carboxyl terminus made

the cells resistant to the effects of TNF $\alpha$  repression of  $\beta$ -catenin signaling as expected, indicating that these domains play an important role in TNF $\alpha$  regulation of  $\beta$ -catenin signaling. Similar experiments were repeated by over-expressing Edar and were surprised to observe that there was a repression of  $\beta$ -catenin signaling activity in all deletion mutants (**Figure 1.20B**). The C-terminus is the transactivation domain, which is responsible for its transcriptional activity and removal of this region results in low reporter activity of  $\beta$ -catenin. It is not surprising to observe that treatment with recombinant TNF $\alpha$  is unable to repress  $\beta$ -catenin signaling further since the intrinsic activity of the deletion mutant is too low to start with. Since cells became resistant to the effects of TNF $\alpha$  after removal of the N-terminus, a further examination was done to determine if there was any change in localization of  $\beta$ -catenin phosphorylated at the N-terminus within the cell after treatment with TNF $\alpha$ . SW480 cells were stained with antibodies that recognize  $\beta$ -catenin only when it is phosphorylated on serines 41 and/or 45. No staining was observed in control cells but a dramatic increase in nuclear staining was observed following treatment with TNF $\alpha$  (**Figure 1.21A-B**). Treatment of SW480 cells with 20ng/ml of TNF $\alpha$  also resulted in an increase in staining using an antibody specific for  $\beta$ -catenin when it is phosphorylated on serines 33, 37 and threonine 41. This staining pattern was cytoplasmic/membrane rather than nuclear (**Figure 1.21C-D**). Similar results were observed in HEK293 cells (not shown). These experiments were performed over two hours and the most obvious difference was observed at thirty minutes (other data not shown). The data here indicate that one or more of the N-terminal serine residues are important for TNF $\alpha$  to repress  $\beta$ -catenin signaling activity. The exact residues involved are unknown but earlier data indicate that phosphorylation sites that

target  $\beta$ -catenin for degradation (Ser 33,37, 45 and Thr41) are probably involved. To formally test this hypothesis, mouse embryonic fibroblasts cells (MEFs) were transfected with  $\beta$ -catenin, mutated either on residues Ser 33, 37, Thr 41 and Ser45 or on serine 45 residue alone. Expression of  $\beta$ -catenin in wild type MEFs was repressed by 55% after treatment with TNF $\alpha$  for sixteen hours (**Figure 1.22A**). In contrast, wild type MEFs transfected with  $\beta$ -catenin mutated on 33, 37, 41 and 45 or on serine 45 alone were resistant to repression by TNF $\alpha$  (**Figure 1.22B-C**). It is interesting to note that the signaling activity increases with the presence of mutated  $\beta$ -catenin after treatment with TNF $\alpha$ . It is possible that there may be co-repressors that bind to  $\beta$ -catenin and are responsible for the repressive effects of TNF $\alpha$  on  $\beta$ -catenin signaling. Mutations on these sites prevent the co-repressor from binding and subsequently inhibiting  $\beta$ -catenin signaling. Taken together these data suggest that serine 45 is a key residue in the regulation of  $\beta$ -catenin repression by TNF $\alpha$  and activated IKK. To specifically test this, another colon cancer cell line, HCT116, which has normal APC but expresses one allele of  $\beta$ -catenin in which serine 45 is deleted and one normal allele, was used. Initial experiments confirmed the ability of TNF $\alpha$  and CAIKKs to down-regulate  $\beta$ -catenin signaling activity in parental HCT116 cells (**Figure 1.23**). Another form of HCT116 cells in which either the normal or the mutated  $\beta$ -catenin allele had been removed by somatic cell knockout was used [235]. Remarkably, cells in which the normal allele had been removed (only expressing mutant  $\beta$ -catenin) were resistant to the effects of TNF $\alpha$  whereas the parental cell line and cells in which the mutant allele was deleted (only expressing normal  $\beta$ -catenin) were sensitive (**Figure 1.24**). Edar was overexpressed in

wild type MEF cells together with different  $\beta$ -catenin point mutants. There was a 50% decrease in signaling activity with wild type  $\beta$ -catenin. Presence of  $\beta$ -catenin mutated at Ser45 also showed a decrease of 49% after Edar overexpression (**Figure 1.25**). However, when all four phosphorylation sites are mutated, the cells become more resistant to the effects of Edar. Unlike earlier findings with  $\text{TNF}\alpha$ , a single mutation of Ser45 residue did not make any difference to the repressive effects of Edar on  $\beta$ -catenin signaling. It seemed to indicate that IKK was the only common point between  $\text{TNF}\alpha$  and Edar repression of  $\beta$ -catenin signaling activity since Edar does not seem to behave in a similar manner to  $\text{TNF}\alpha$ . A recent paper by Chaudhary et al. showed that the death domain of Edar is required to activate NF $\kappa$ B, JNK, and caspase-independent cell death pathways and that these activities are impaired in mutants lacking its death domain or those associated with anhidrotic ectodermal dysplasia and the downless phenotype [169]. A schematic of the different Edar deletion constructs, including the wild type is shown in

**Figure 1.25A.** **Figure 1.25B** shows the activity of these mutants on NF $\kappa$ B reporter activity. Another approach was taken to determine if the death domain of Edar may be involved in regulating Edar repression on  $\beta$ -catenin signaling (**Figure 1.25C**). Results indicate that the region within the intracellular domain of Edar that includes the death domain (amino acid 225–448), is required to repress  $\beta$ -catenin signaling activity. It is interesting that to note that autosomal recessive mutation, E379K has little NF $\kappa$ B activity and still represses  $\beta$ -catenin signaling. This confirms the independence of NF $\kappa$ B and  $\beta$ -catenin signaling pathways. Also, the autosomal dominant mutation, R420Q still has significant  $\beta$ -catenin signaling activity and yet both point mutations produce similar phenotypes, indicating that possibility that  $\beta$ -catenin signaling is not involved in

ectodermal dysplasia. However, more deletion mutants and sequence analysis would have to be done in order to determine the exact region required for repressing  $\beta$ -catenin signaling. This chapter provides evidence that NF $\kappa$ B activation is not required for the TNF $\alpha$ -mediated repression in  $\beta$ -catenin signaling confirming that 'cross-talk' between the 2 pathways does not occur downstream of transcription factor activation. However, it should be noted that RelA (-/-) mice models were not used to confirm this and as stated earlier, the concentration of RNA oligonucleotide used was rather high (80nM), and deleterious side effects may occur. Instead, experiments should be repeated in knockout models to confirm these observations. However, p65 (-/-) mice do not have any obvious Wnt phenotype. It was also shown that neither TNF $\alpha$  nor Edar reduce total  $\beta$ -catenin protein levels. Recent evidence using knockout mice models have showed that IKK $\alpha$  is involved in skin differentiation. There was an increase in nuclear  $\beta$ -catenin staining in IKK $\alpha$  (-/-) knockouts and this is consistent with the hypothesis that IKK is involved in down-regulation of  $\beta$ -catenin.

The use of phosphospecific antibodies show that TNF $\alpha$  influences the phosphorylation status of  $\beta$ -catenin in SW480 cells. Consistent with the reporter assay, we observed that TNF $\alpha$  treatment of cells leads to a significant reduction in nuclear  $\beta$ -catenin de-phosphorylated on residues Ser37 and Thr41 clearly suggesting that  $\beta$ -catenin signaling activity seems to depend on the presence of a small pool of transcriptionally active  $\beta$ -catenin that is not phosphorylated on residues Ser 37 and Thr 41. Also,  $\beta$ -catenin phosphorylated at Ser 33, Ser37 and Thr41 was increased in the cytoplasm after 30min treatment with TNF $\alpha$  and  $\beta$ -catenin phosphorylated at Thr41 and Ser45 was increased in the nucleus after the same period of time. These distinct staining patterns disappeared

after 30min indicating that these phosphorylated  $\beta$ -catenin proteins have a short half-life and are otherwise hard to detect. It is logical to assume that there would be an increase in phosphorylated  $\beta$ -catenin at Ser 33, Ser37 and Thr41 in the cytoplasm and not nucleus since this targets  $\beta$ -catenin for ubiquitin-mediated degradation. Serine 45 primes  $\beta$ -catenin for further phosphorylation on other residues and the rapid increase of  $\beta$ -catenin phosphorylated on Ser45, in the nucleus possibly indicates that the 'active' fraction of  $\beta$ -catenin is being exported out of the nucleus into the cytoplasm. On the other hand, we did not observe any increase in staining of phosphorylated  $\beta$ -catenin at Thr41 and Ser45 in the cytoplasm. Thus our results indicate that even though treatment with TNF $\alpha$  results in changes in phosphorylation of  $\beta$ -catenin, this phosphorylation does not target the overall pool of  $\beta$ -catenin for degradation within the cell.

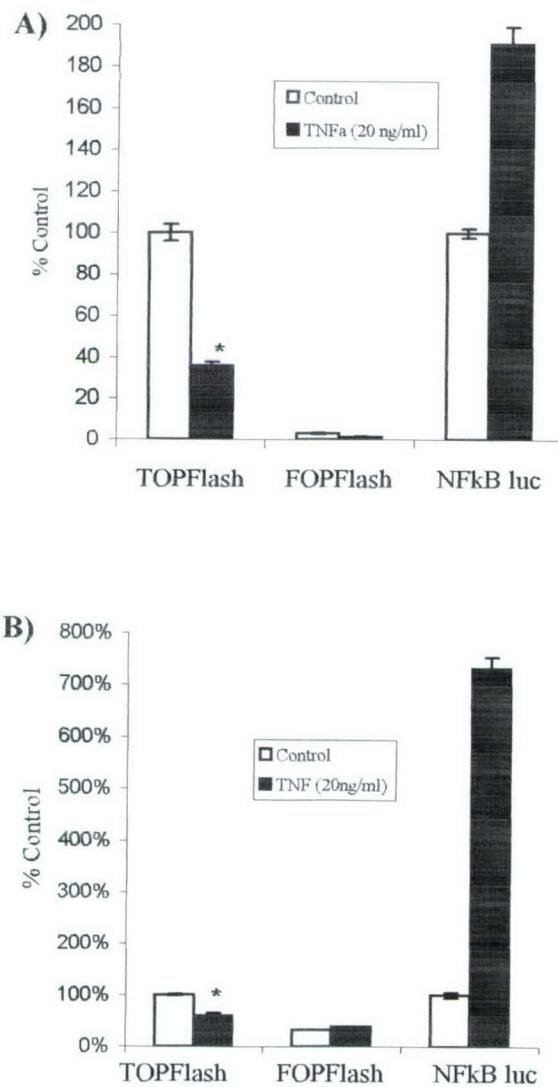


Figure 1.1: Effect of TNFa on b-catenin signaling. A) SW480 cells and B) HEK293 cells were transfected with 0.1 $\mu$ g of reporter, Topflash or NFkB and incubated for twenty-four hours. The cells were then treated with 20ng/ml of TNFa for a further sixteen hours. Cells were harvested and the luciferase activity assayed. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

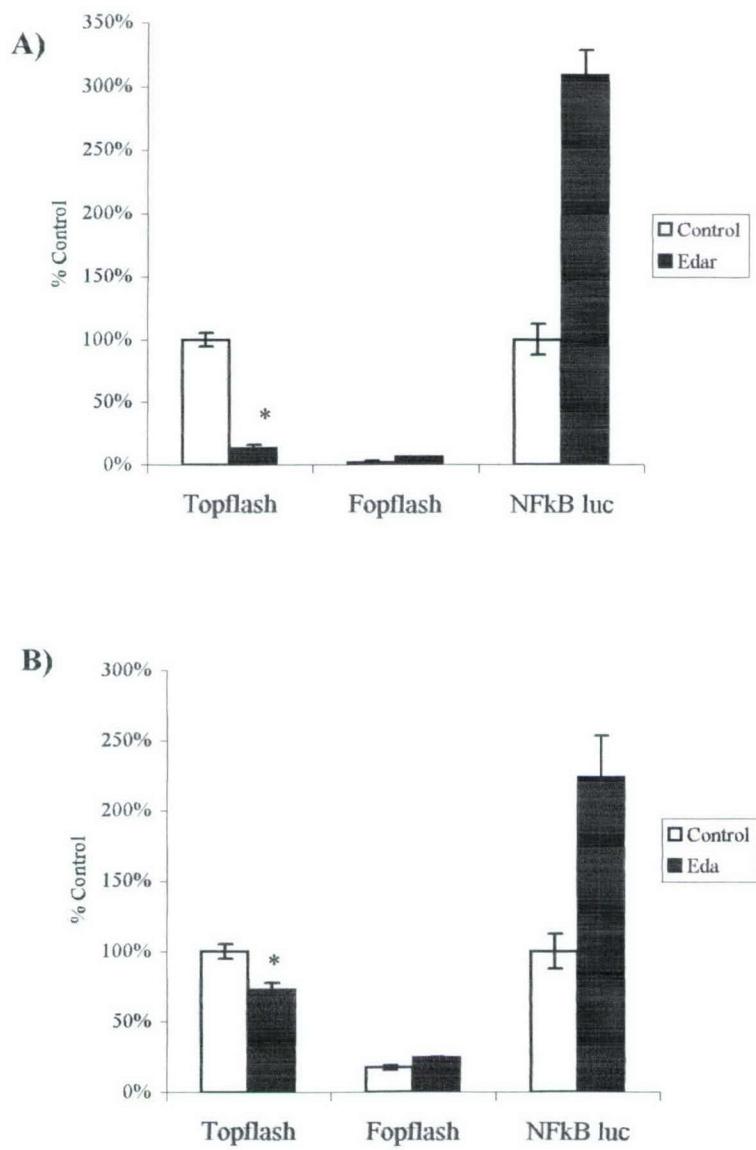


Figure 1.2: Effect of Eda/Edar on b-catenin signaling. A) SW480 cells were transfected with 50ng of ectodysplasin receptor, Edar together with 0.1ug of either Topflash or NFkB reporter and allowed to incubate for twenty-four hours. B) SW480 cells were transfected with 50ng Eda and a similar amount of reporters for twenty-four hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

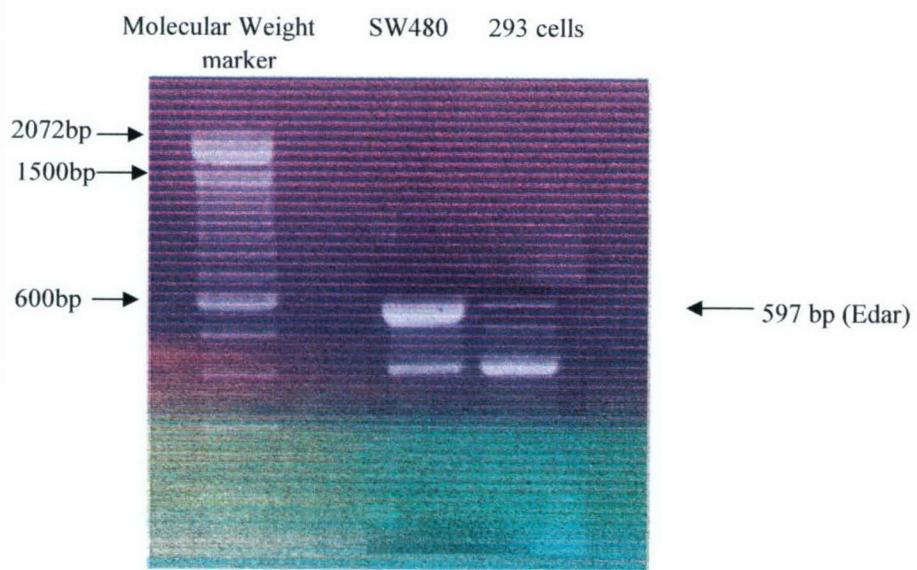


Figure 1.3: Expression of Edar in SW480 and HEK293 cells. RNA was harvested from 2 cell types, SW480 and HEK293 cells as described in Materials and Methods section. One-step PCR was performed and the product was run on 2% agarose gel together with 100bp molecular weight marker. A band corresponding to Edar is observed on the gel at around 600bp and is indicated by arrow. There is another unknown band about 400bp in size.

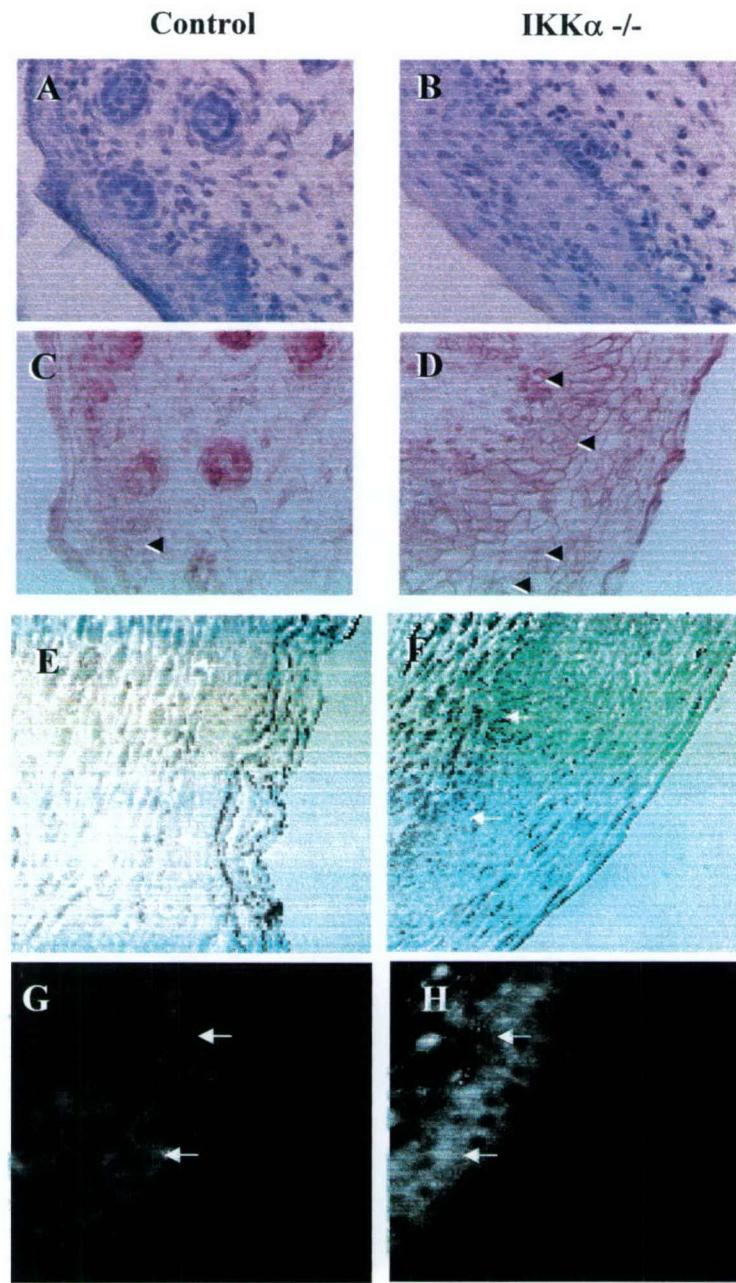


Figure 1.4:  $\beta$ -catenin and cyclin D1 are localized in the nuclei of epidermal cells in IKK $\alpha$  -/- mouse. Both wildtype (WT) (A) and IKK $\alpha$  -/- (B) embryos were stained with mouse IgG and hematoxylin. No background staining was observed with the IgG control antibody. Note the thickened epidermis of the IKK $\alpha$  -/- embryo. Because hematoxylin masked the nuclear  $\beta$ -catenin staining, the embryos were stained with  $\beta$ -catenin alone. Membrane staining was observed in both WT (C) and IKK $\alpha$  -/- (D). However, an increased number of epidermal cells with nuclear  $\beta$ -catenin (arrows) was observed in the IKK $\alpha$  -/- mouse. Cyclin D1 expression was also increased in the basal aspects of the epidermis of the IKK $\alpha$  -/- mouse. (E-H) Cyclin D1 staining was performed in Richard Pestell's laboratory

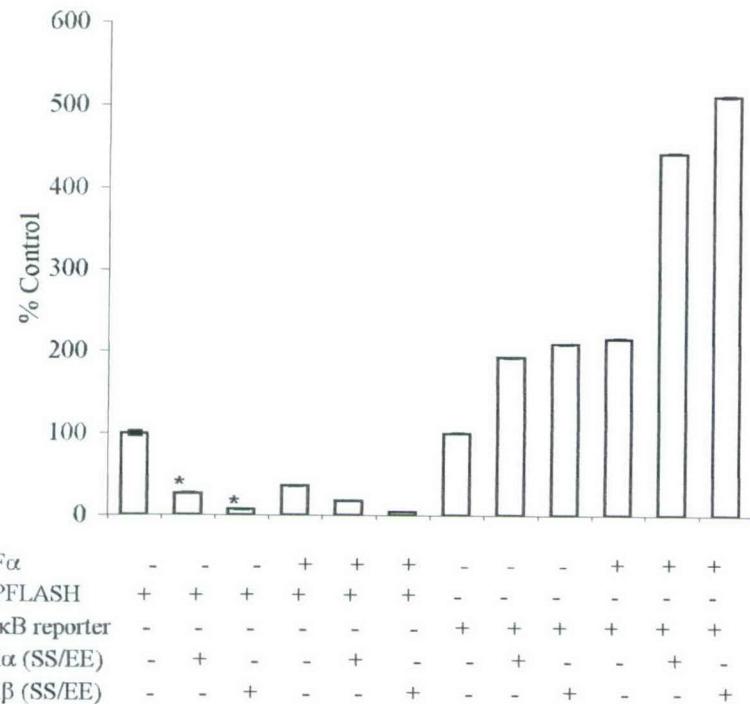


Figure 1.5: Effect of constitutively active IKK $\alpha$  and IKK $\beta$  mutants on b-catenin signaling. SW480 cells were transfected with either 0.1ug of Topflash or NF $\kappa$ B reporter and 0.5ug of constitutively active IKK mutants. After twenty four hours, they were treated with 20ng/ml of TNF $\alpha$  for a further sixteen hours and harvested. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments,  $\pm$  standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

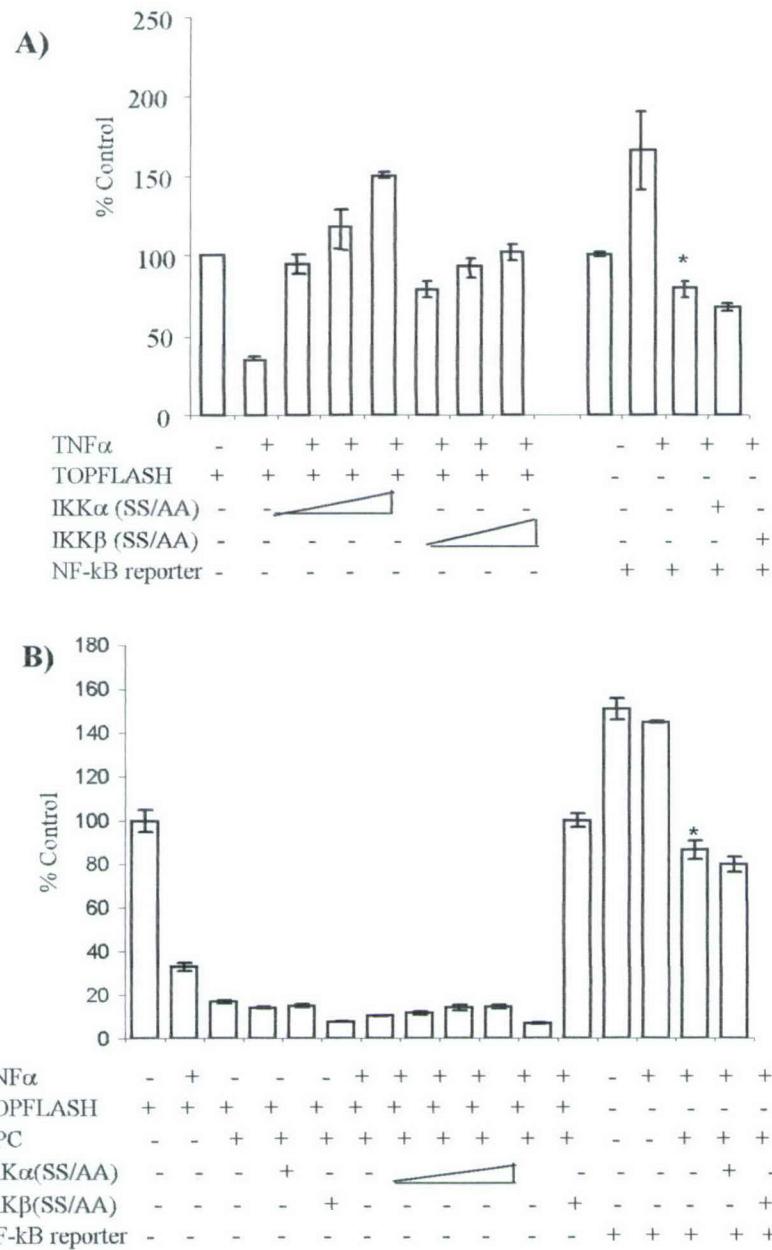
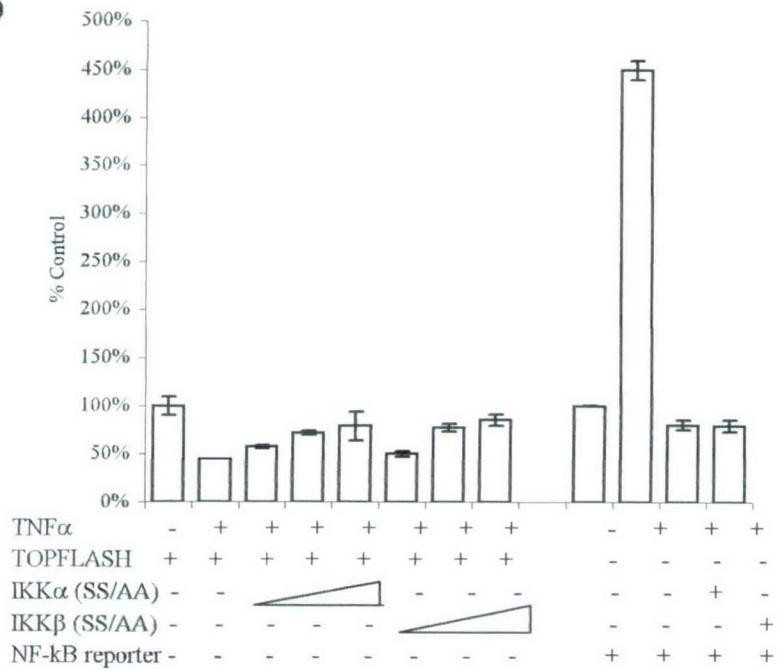


Figure 1.6: Effect of dominant negative IKK mutants (DN-IKK) and APC on  $\square$ -catenin signaling. A) SW480 cells were transfected with 0.1ug of Topflash reporter and 0.1ug, 0.5ug and 1.0ug of DN-IKK mutants. When NF $\square$ B was used, 1.0ug of DN-IKK mutants were used. After twenty-four hours, cells were treated with 20ng/ml of TNF $\square$  for sixteen hours, after which they were harvested. B) Similar experiments were carried with the addition of 0.5ug of wild type APC. C) Similar experiments were repeated in HEK293 cells. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

C



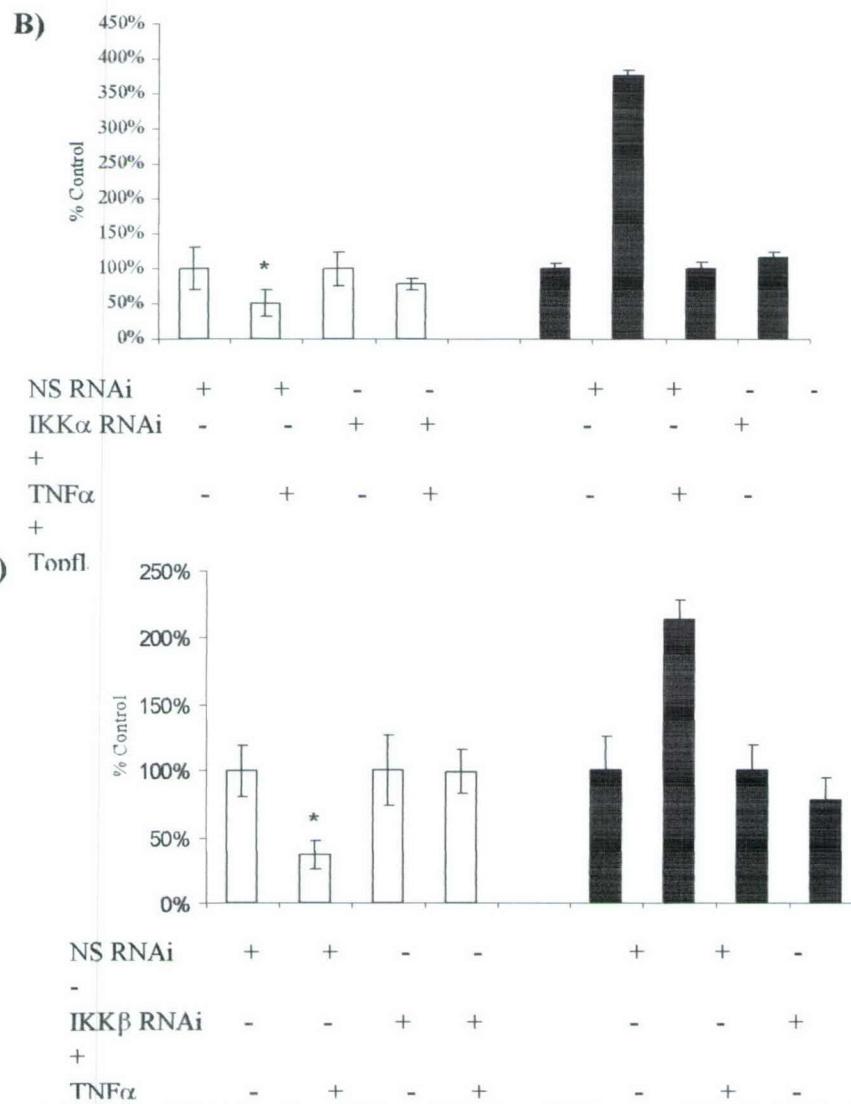
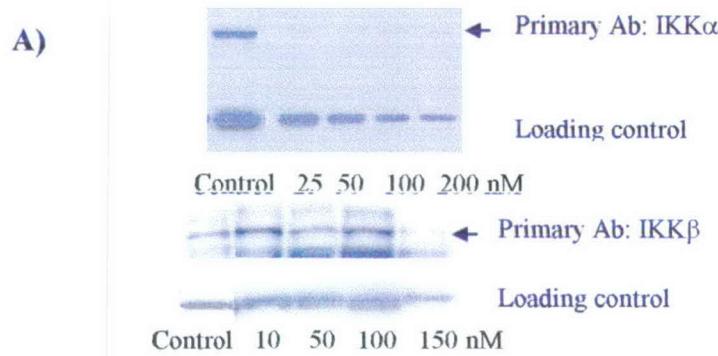


Figure 1.7 Effect of RNAi against IKK $\alpha$  and IKK $\beta$  on TNF $\alpha$  treated SW480 cells. A) SW480 cells were treated with increasing doses of RNAi against IKK $\alpha$  and IKK $\beta$  (from 25nM to 200nM) over seventy-two hours. Media was changed and cells were treated with 20ng/ml of TNF $\alpha$  for a further sixteen hours and whole cell lysates were made. (see in Materials and Methods). The top panel shows that the band corresponding to IKK $\alpha$  is decreased after treatment with 25nM of IKK $\alpha$  RNAi. The bottom panel shows that the band corresponding to IKK $\beta$  decreases slightly after treatment with 150nM of IKK $\beta$  RNAi. B) Luciferase reporter assay. SW480 cells were treated with controls which were non-specific RNAi (NSRNAi) or 25nM of RNAi targeted against IKK $\alpha$  over seventy-two hours and transiently transfected with 0.1ug Topflash or NF $\kappa$ B reporter for twenty-four hours. C) As B but using 100nM of RNA targeted against IKK $\beta$ . Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

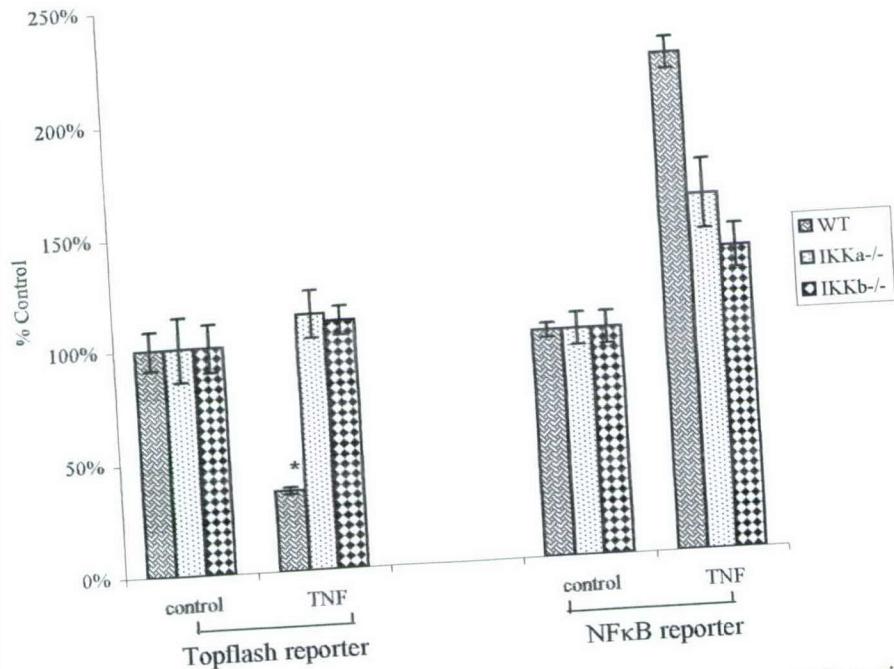


Figure 1.8: Effect of TNF $\alpha$  on b-catenin signaling in IKK $(-/-)$  mouse embryonic fibroblast (MEF) cells. Wild Type MEF cells, IKK $\alpha$  $^{-/-}$  and IKK $\beta$  $^{-/-}$  cells were transfected with 0.1ug of Topflash or NF $\kappa$ B reporter for twenty four hours and treated with 20ng/ml of TNF $\alpha$  for a further sixteen hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

A)

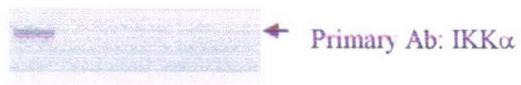
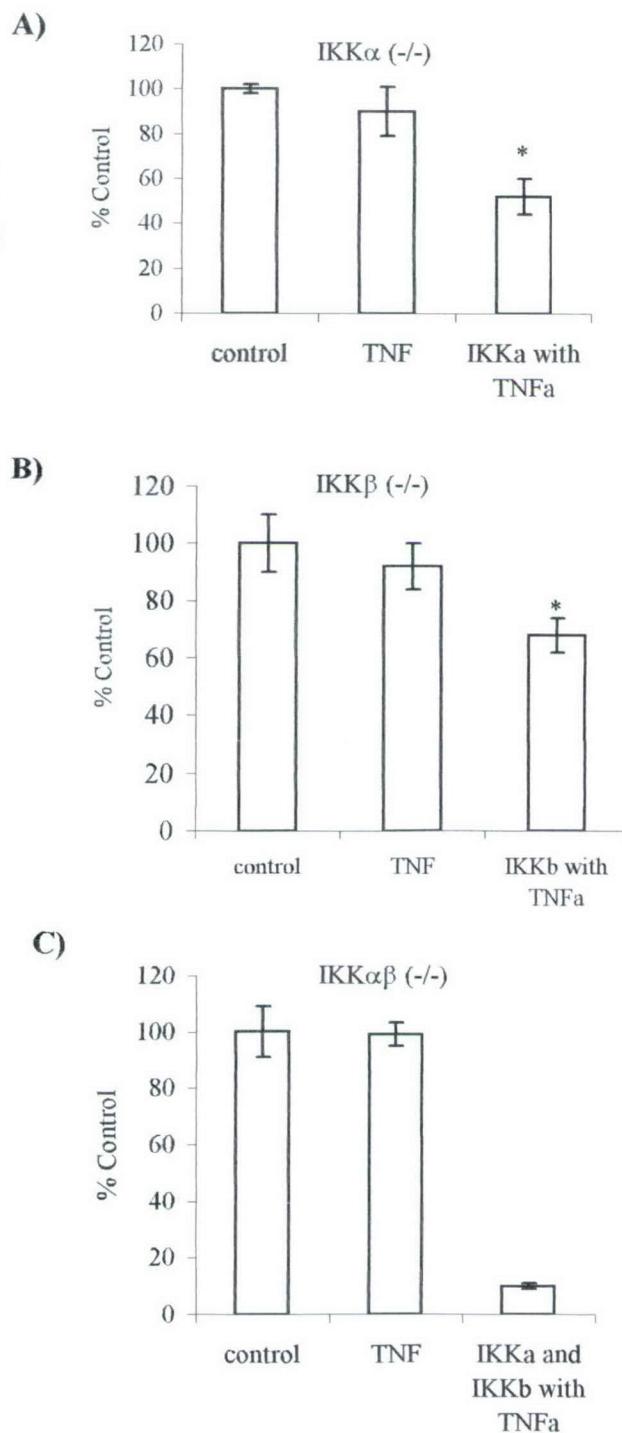


Figure 1.9: Effect of wild type (WT) IKK $\alpha$  and IKK $\beta$  on TNF $\alpha$  regulation of  $\beta$ -catenin signaling in MEF cells. A and D) IKK $\alpha$  (-/-) B and E) IKK $\beta$  (-/-) C and F) double knockouts IKK $\alpha\beta$  (-/-) MEFs transfected with 0.1ug of Topflash reporter (A,B,C) or NF $\kappa$ B reporter (D,E,F) and 0.5ug of WT IKKs. After twenty-four hours, they were treated with 20ng/ml TNF $\alpha$  for sixteen hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .



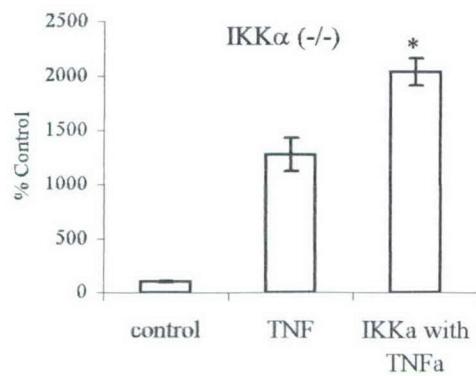
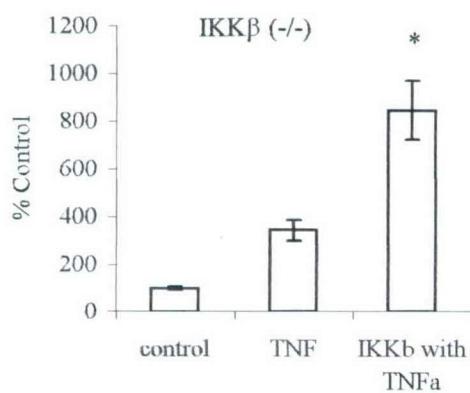
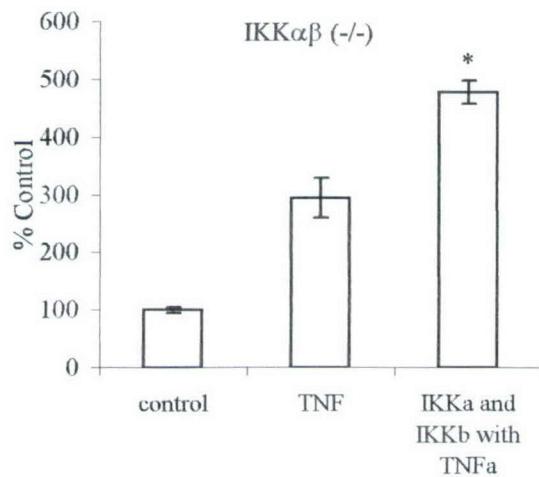
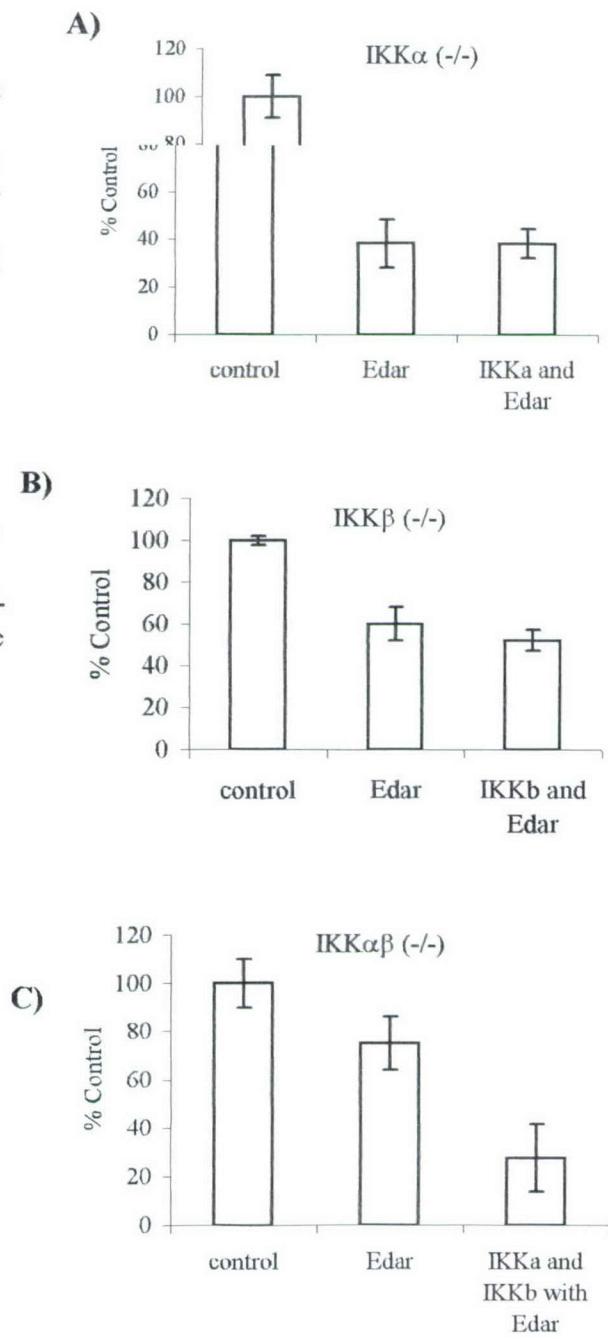
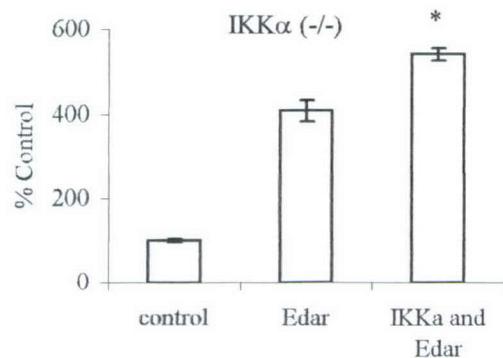
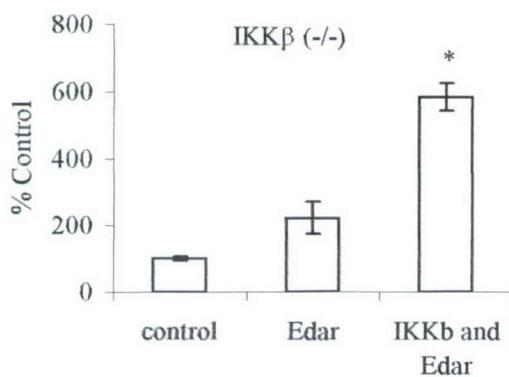
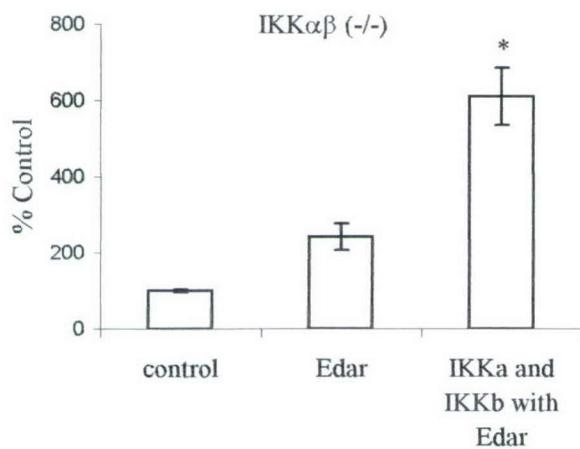
**D)****E)****F)**

Figure 1.10 Effect of WT IKK on Edar regulation of  $\beta$ -catenin signaling in IKK $\alpha$  (-/-) MEF cells. A and D) IKK $\alpha$  (-/-) B and E) IKK $\beta$  (-/-) C and F) double knockouts IKK $\alpha\beta$  (-/-) MEFs were transfected with 0.1ug Topflash reporter (A,B,C) or NF $\kappa$ B reporter (D,E,F), 50ng Edar and 0.5ug each of wild type IKKs for forty-eight hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .



**D)****E)****F)**

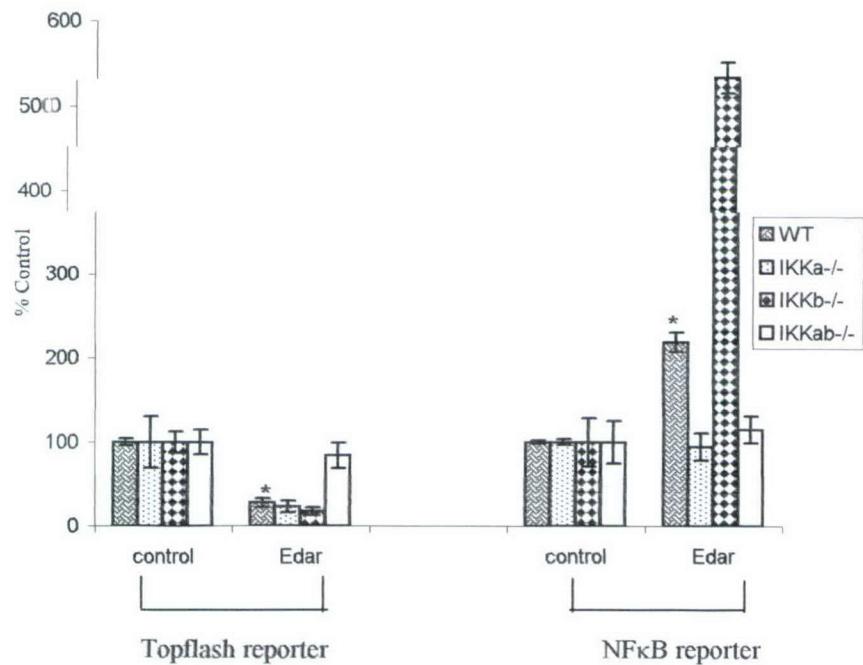
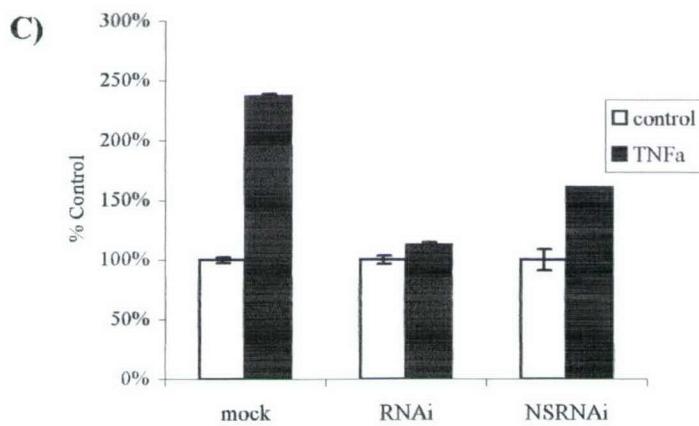
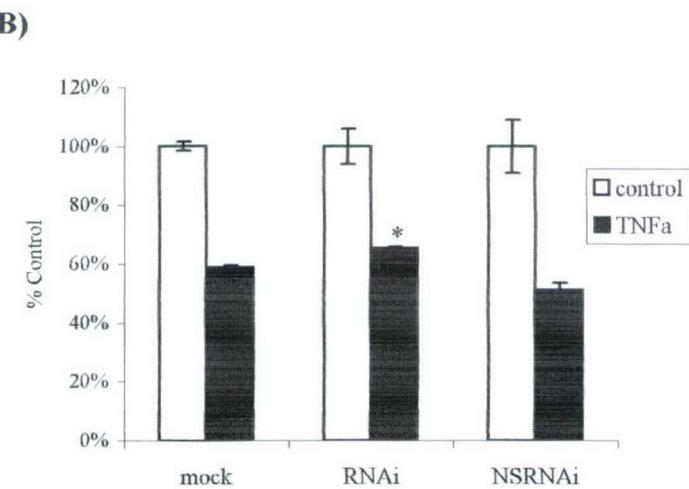
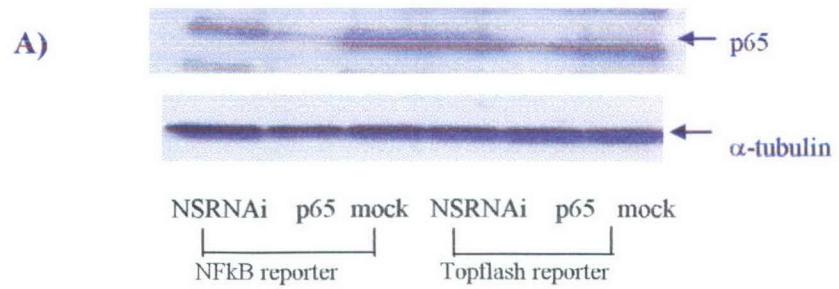
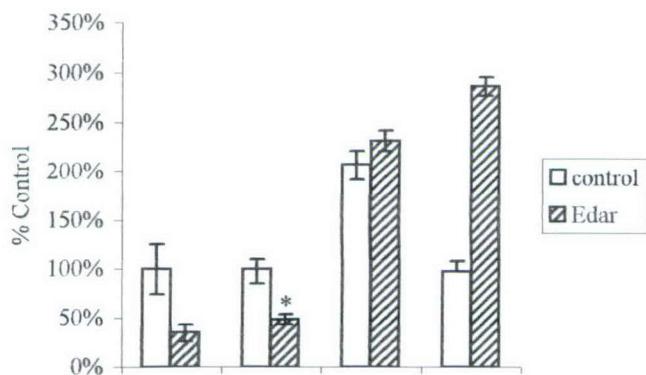


Figure 1.11 Effect of Edar on b-catenin signaling activity in IKK (-/-) MEF cells. Wild type and knockout MEF cells were transfected with 50ng of Edar together with 0.1ug of reporter for forty-eight hours. Luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .



D)



Topflash	+	+	-	-
NFkB	-	-	+	+
p65 RNAi	-	+	+	-
NS RNAi	+	-	-	+

Figure 1.12 Influence of NF- $\kappa$ B activity on TNF $\alpha$ /Edar repression of b-catenin signaling. A) Western Blot. SW480 cells were treated with 80nM RNAi targeted against active NFkB, p65 subunit for seventy-two hours, harvested and lysed as described in Materials and Methods. Loading controls with a-tubulin are shown at the bottom panel. B) Topflash reporter assay. SW480 cells were treated with 80nM RNAi targeted against active NFkB, p65 subunit for seventy-two hours and transfected with 0.1ug of reporters, Topflash or NFkB for twenty four hours. 20ng/ml of TNF $\alpha$  was then added for sixteen hours. Cells were harvested and luciferase activity was measured. Mock transfections denote the use of transfecting reagent, Oligofectamine in the absence of RNAi and cells were treated the same way. Scrambled RNAi (NSRNAi) was also used as a control. The sequence is described in Materials and Methods. C) NFkB reporter assay. Cells were treated in the same way as described in the Topflash assay. D) SW380 cells were treated with non specific RNAi (NSRNAi) and RNAi against p65 subunit as B). However 50ng of Edar was transfected together with the reporters for twenty-four hours. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

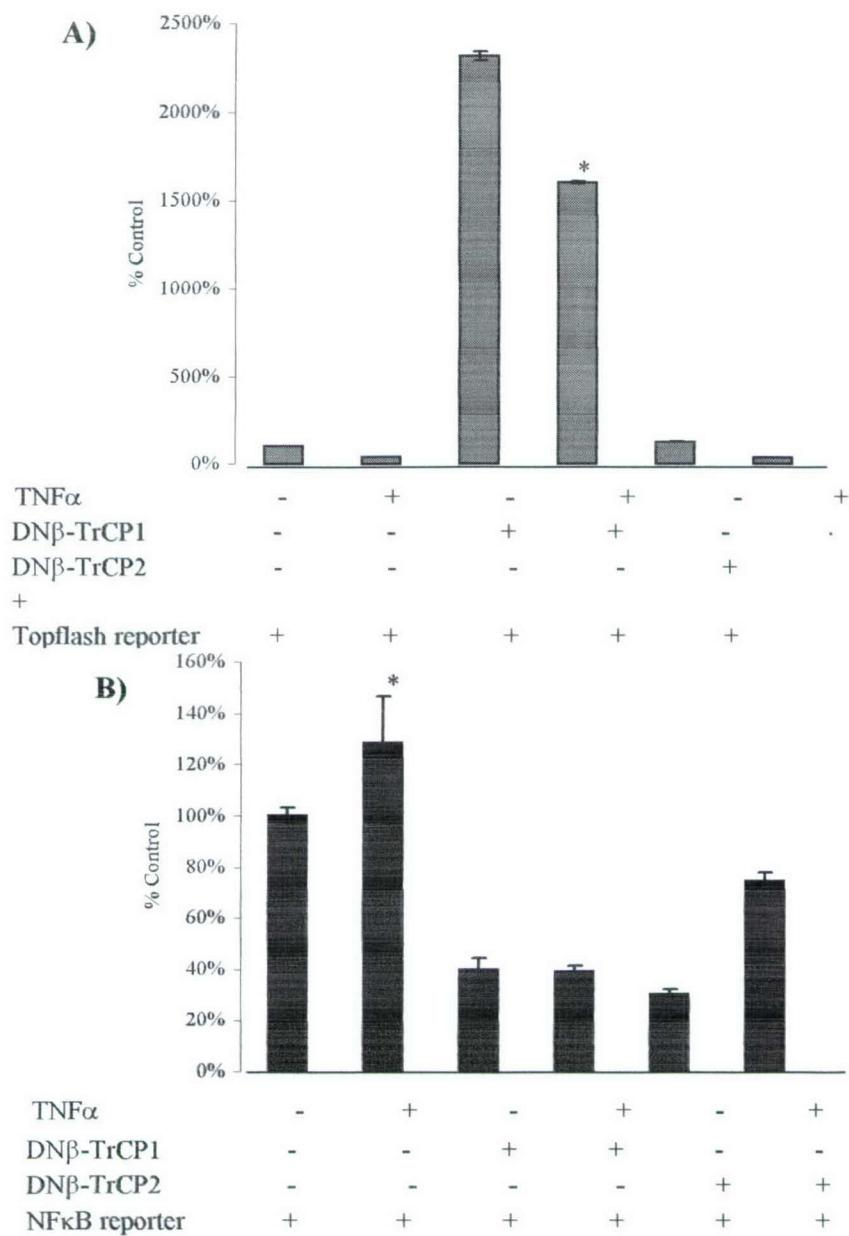


Figure 1.13 Effect of dominant negative b-TrCP (DN b-TrCP) on TNF $\alpha$  regulation of b-catenin signaling activity. A) Topflash reporter assay in HEK293 cells. 0.5ug of DN b-TrCP1 and 0.1ug of Topflash reporter were transfected into 293 cells for twenty-four hours. After which, 20ng/ml of TNF $\alpha$  was added for sixteen hours. Cells were harvested and luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ . B) NF $\kappa$ B reporter assay. Experiment was repeated as in A) with 0.1ug of NF $\kappa$ B reporter transfected instead.

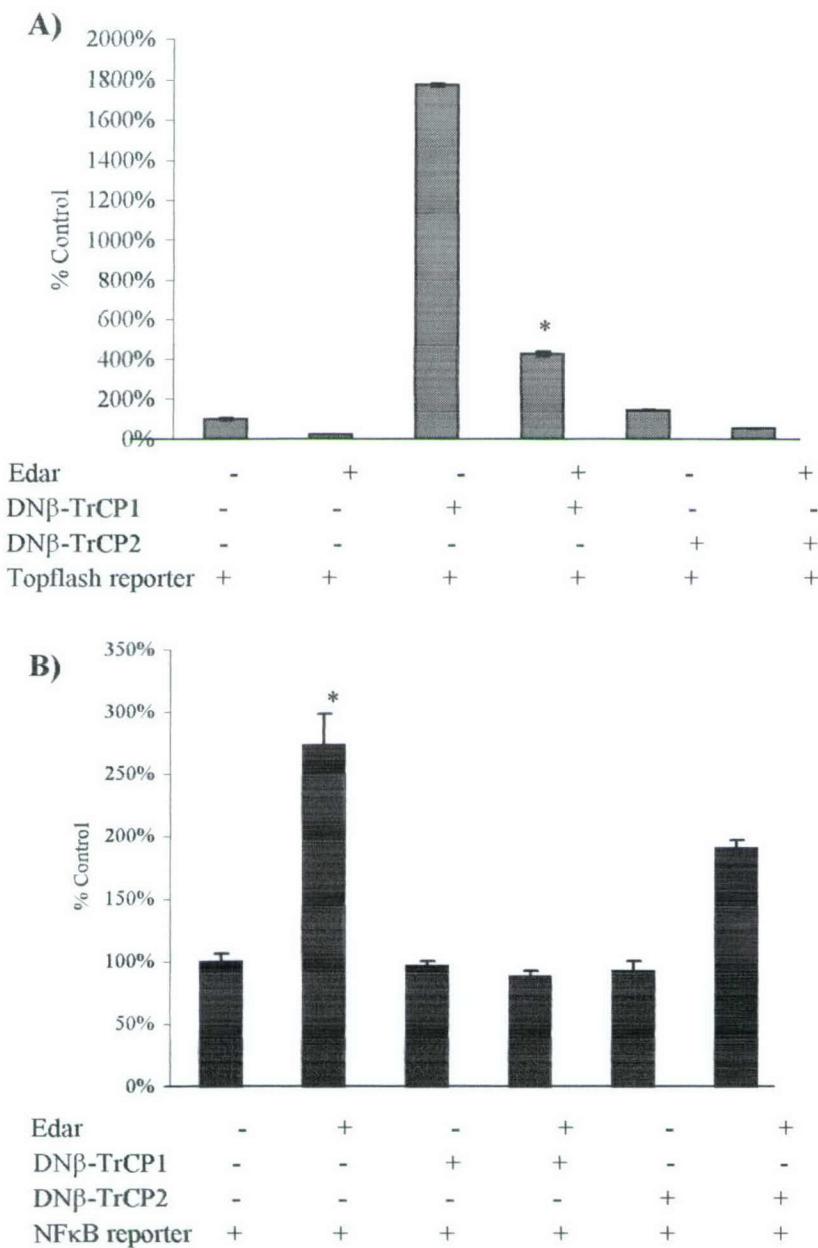


Figure 1.14 Effect of dominant negative b-TrCP (DN b-TrCP) on Edar regulation of b-catenin signalling activity. A) Topflash reporter assay in HEK293 cells. 0.5ug of DN b-TrCP1, 0.1ug of Topflash reporter and 50ng of Edar were transfected into 293 cells for twenty-four hours. Cells were harvested and luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ . B) NF $\kappa$ B reporter assay. Experiment was repeated as in A) with 0.1ug of NF $\kappa$ B reporter transfected instead.

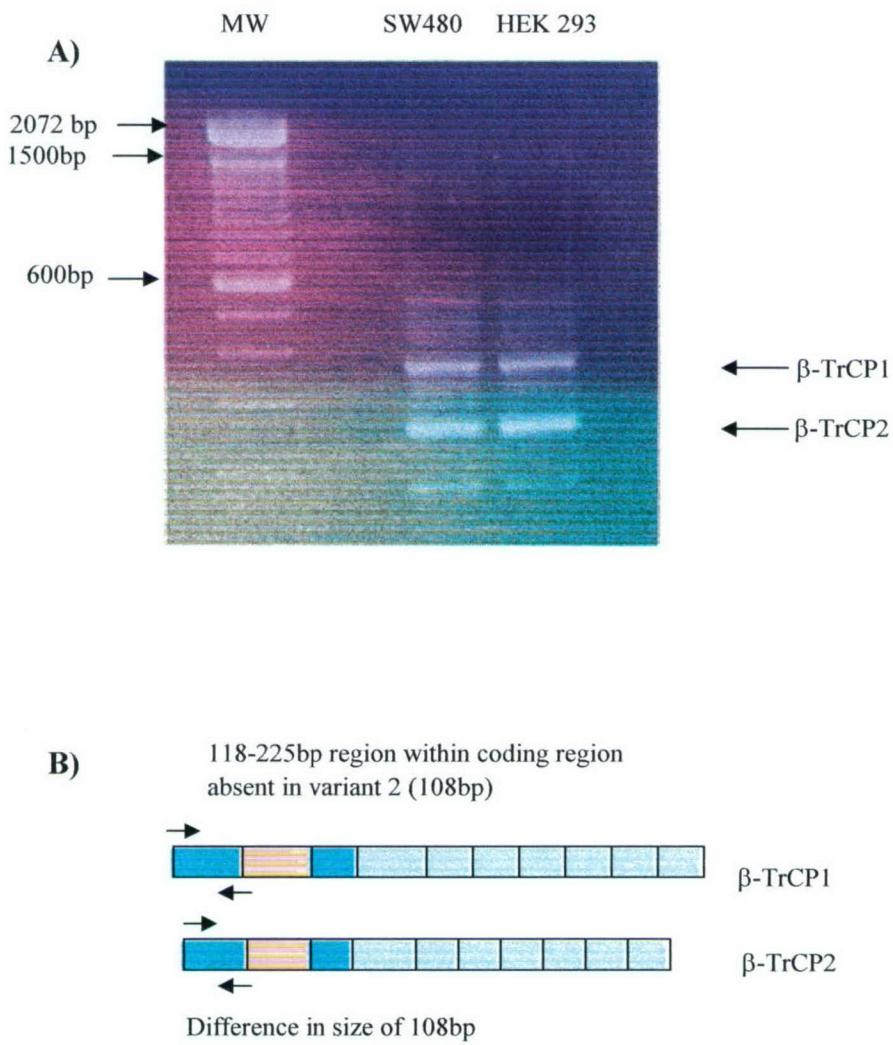


Figure 1.15 Expression of b-TrCP in SW480 and HEK293 cells. A) RT-PCR of SW480 cells and HEK 293 cells. RNA were harvested from cells as described in Materials and Methods. 2ul of PCR product together with loading buffer, was loaded onto 1% agarose gel and allowed to run for one hour at 80V. There are 2 bands seen for each cell line, indicating that both F-box proteins, b-TrCP1 (364bp) and b-TrCP2 (256bp) are present. 100bp molecular weight marker (MW) is shown on extreme left. B) Schematic diagram of the location of the primers for each of the F-box protein. Similar primers were chosen for each protein. However, due to the absence of a 108 bp fragment in b-TrCP2, a size difference was observed.

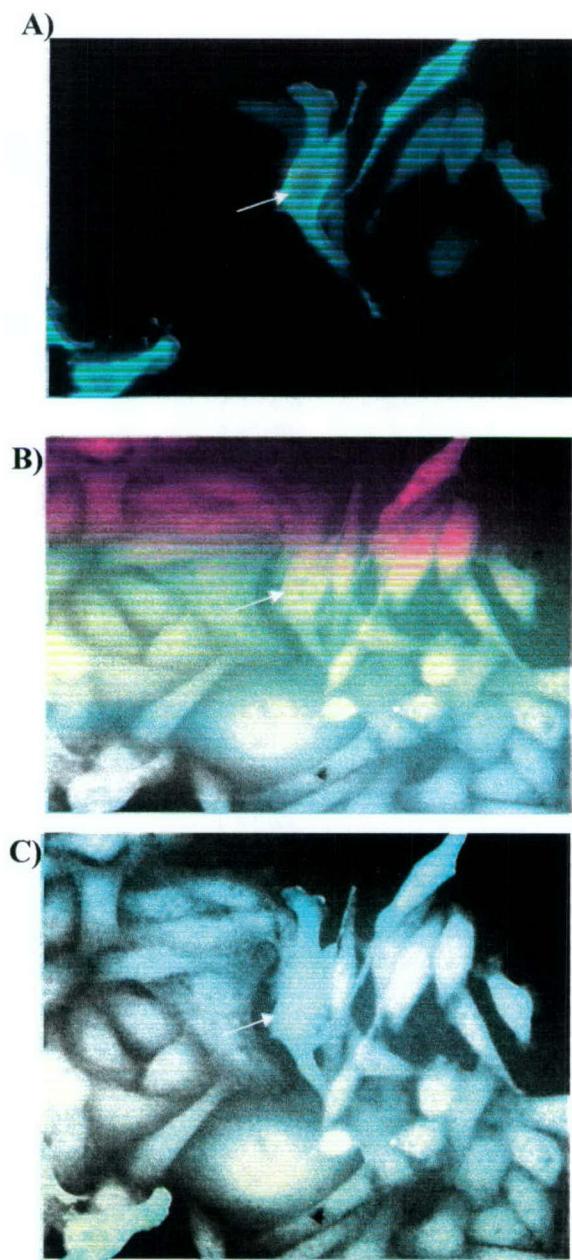


Figure 1.16 Effect of constitutively active IKK (CA IKK) mutants on localization of total  $\beta$ -catenin. A) SW480 cells were transiently transfected with 1.0ug of flag-tagged CA IKK $\alpha$  for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. Staining was performed first using polyclonal flag antibody and then fluorescein-conjugated secondary antibody (green) (transfected cells indicated by arrow). B) Cells were stained for total  $\beta$ -catenin using monoclonal C-terminus  $\beta$ -catenin antibody and then Texas Red-conjugated secondary antibody. (red) (same cell transfected with CA IKK mutant indicated by arrow) C) A superimposed image of both A) and B).

Figure 1.17 Effect of TNFa on localization of de-phosphorylated b-catenin in SW480 cells. A) Control SW480 cells without treatment with TNFa. B) SW480 cells were treated with 20ng/ml TNFa for sixteen hours and monoclonal aABC (de-phosphorylated b-catenin) antibody and fluorescein-conjugated secondary antibody were used. Staining was performed as described in Materials and Methods. Pictures were taken at similar exposure times (indicated by green staining) C) Statistical analysis of cells with no nuclear staining before and after treatment with TNFa. 7 fields of view at 200X magnification were randomly selected and the number of cells were counted and the average calculated. Statistical analysis was performed using paired Student's T-test. \* indicates statistical significance between treatments, where  $p < 0.05$ .

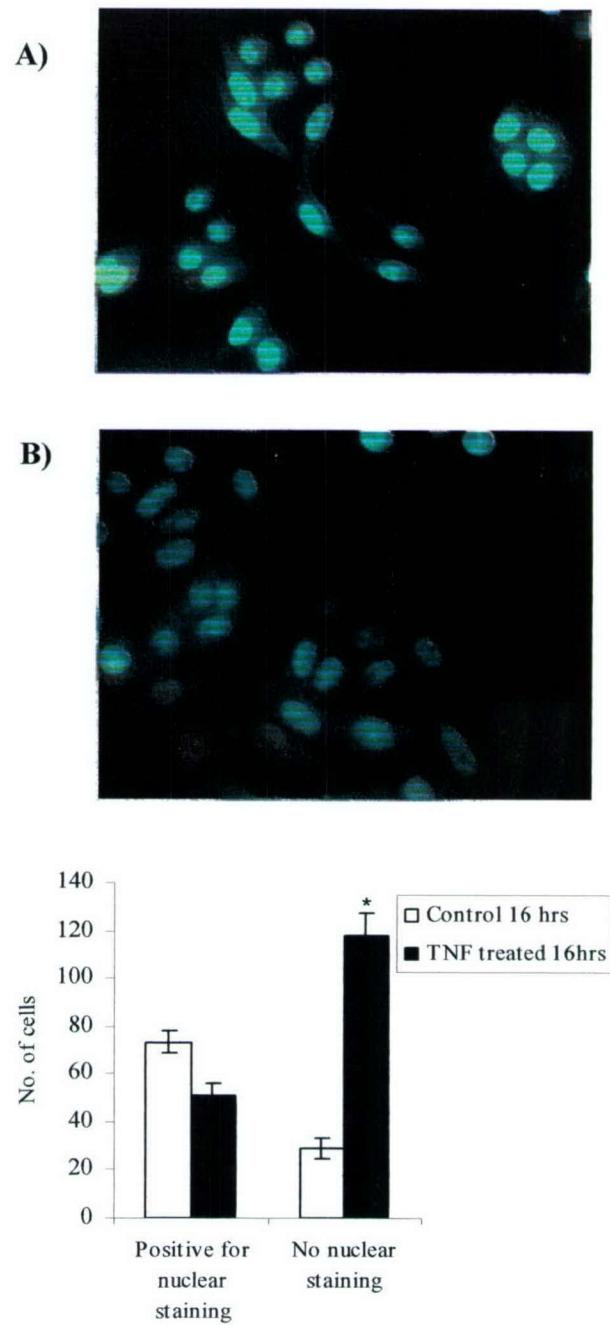


Figure 1.18: Effect of over-expression of Edar on localization of de-phosphorylated  $\beta$ -catenin protein in SW480 cells. SW480 cells were transfected with 50ng of flag-tagged Edar for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. A) SW480 cells stained with polyclonal anti-flag antibody and Texas red-conjugated secondary antibody (cells transfected with Edar are stained red and denoted by arrow) B) Same field of view showing cells stained with fluorescein-conjugated monoclonal aABC (cells are stained green). C) Superimposed image of A) and B). Same arrow denotes cells that are transfected with Edar do not have any nuclear staining. D) Statistical analysis of the number of cells with nuclear staining. Average cell counts of 7 field of views at 200X magnification for control (cells not transfected) and cells transfected with Edar. Statistical analysis was performed using paired Student's T-test. \* indicates statistically significant, where  $p < 0.05$ .

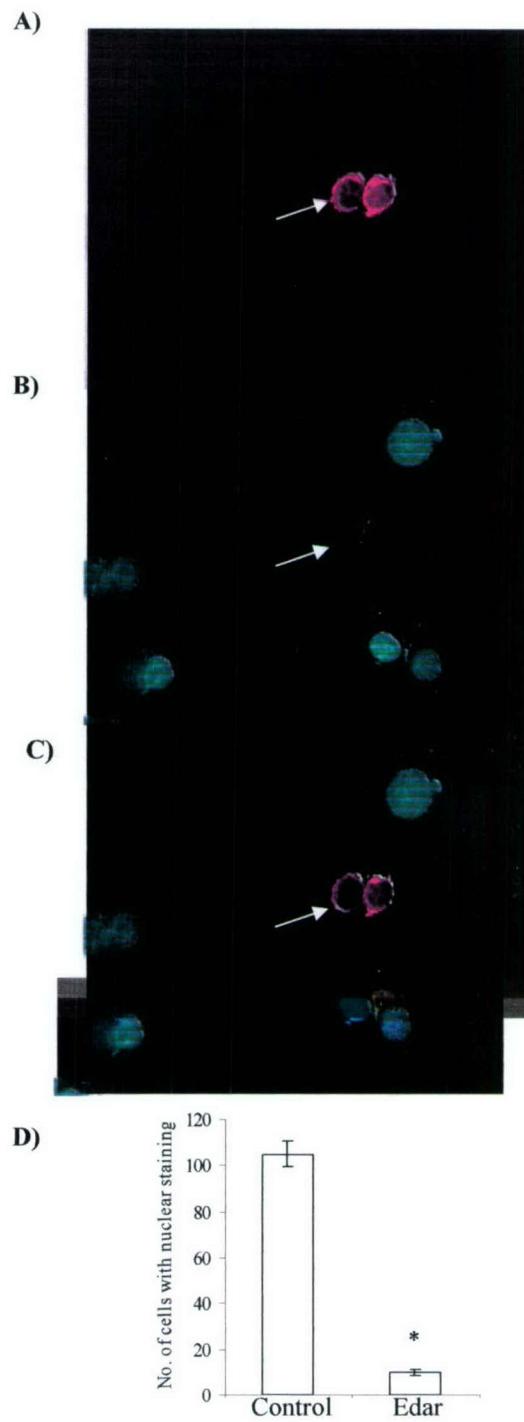
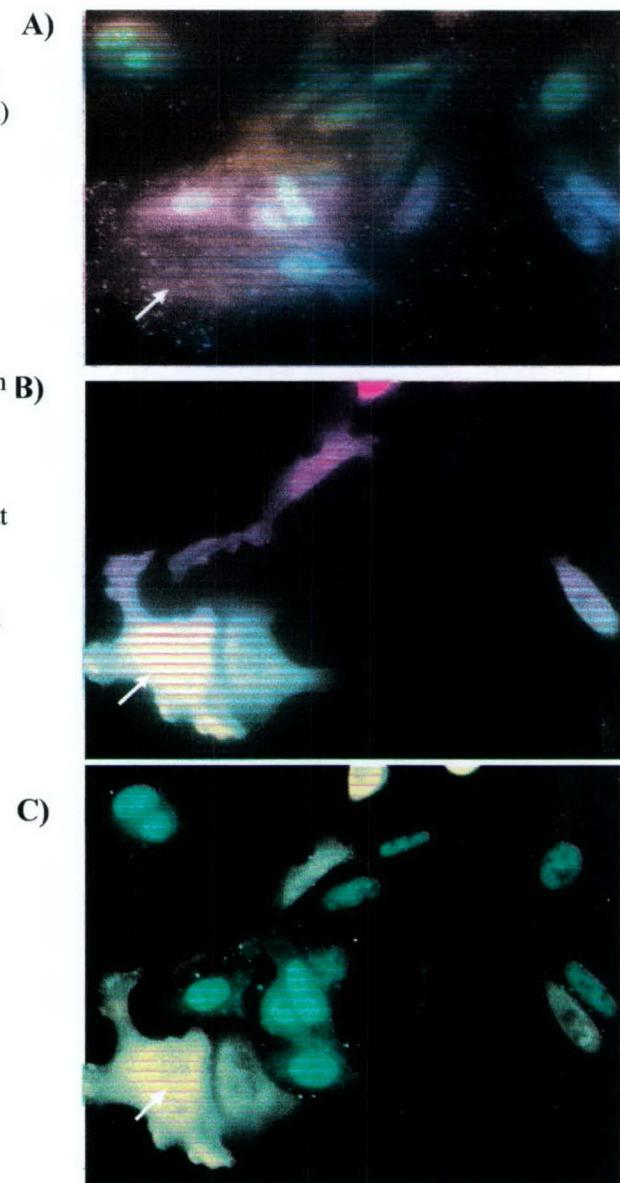
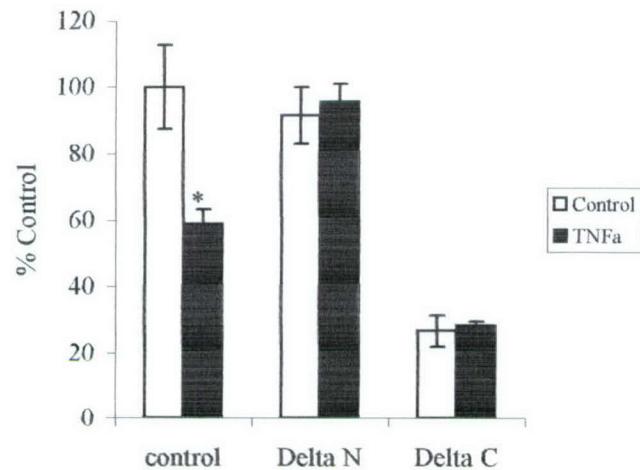


Figure 1.19 Effect of over-expression of constitutively active IKK (CA IKK) mutants on localization of de-phosphorylated  $\beta$ -catenin in SW480 cells. SW480 cells were transfected with 1.0ug of CA IKK $\alpha$  for twenty-four hours. Cells were fixed and stained as described in Materials and Methods. A) Cells were first probed with polyclonal flag antibody and then B) stained using Texas Red-conjugated secondary antibody (red). B) Cells were stained using antibody directed against  $\beta$ -catenin de-phosphorylated at Ser37, Thr41 (green). Cells transfected with IKK mutant did not have any nuclear staining of  $\beta$ -catenin (indicated by arrow) when compared to control (cells without IKK). C) superimposed image of A) and B)



A)



B)

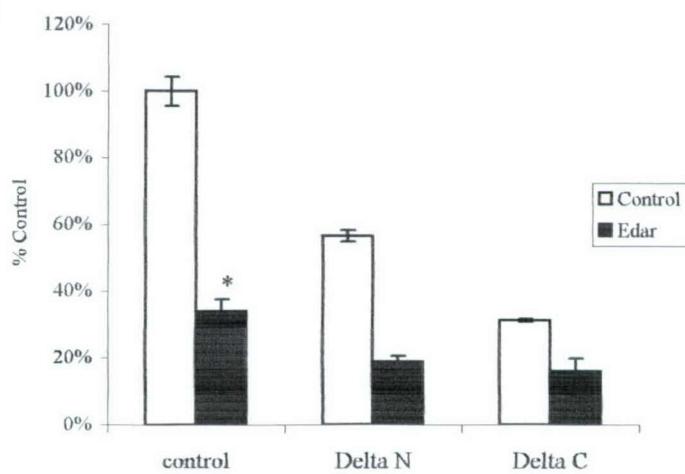


Figure 1.20 Effect of TNFa on truncated forms of b-catenin. A) Wild type MEF cells were transfected with 0.1ug of Topflash reporter and 0.5ug of either wild type b-catenin or deletion mutants of b-catenin for twenty-four hours. Cells were treated with 20ng/ml TNFa for an additional sixteen hours and luciferase activity was measured. Mutant forms of b-catenin, delta N (truncated N-terminus), delta C (truncated C-terminus) were made via PCR and their primers are described in Materials and Methods. B) Similar experiments were carried out with over-expression of 0.1ug of Topflash reporter, 50ng of Edar and 0.5ug of different forms of b-catenin. Cells were incubated for twenty-four hours. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

Figure 1.21 Localization of phosphorylated forms of b-catenin in SW480 cells after TNFa treatment. SW480 cells were treated with 20ng/ml of TNFa for 30 mins. Cells were fixed and stained as described in Materials and Methods. A) Cells without treatment (controls) were stained with antibody that recognizes b-catenin phosphorylated at Thr41/Ser45. B) Similar staining was performed on cells treated with TNFa. C) Cells without treatment (controls) were stained with antibody that recognizes b-catenin phosphorylated at Ser33, Ser37, Thr41. D) Similar staining was done with cells treated with TNFa. Experiments were repeated three times.

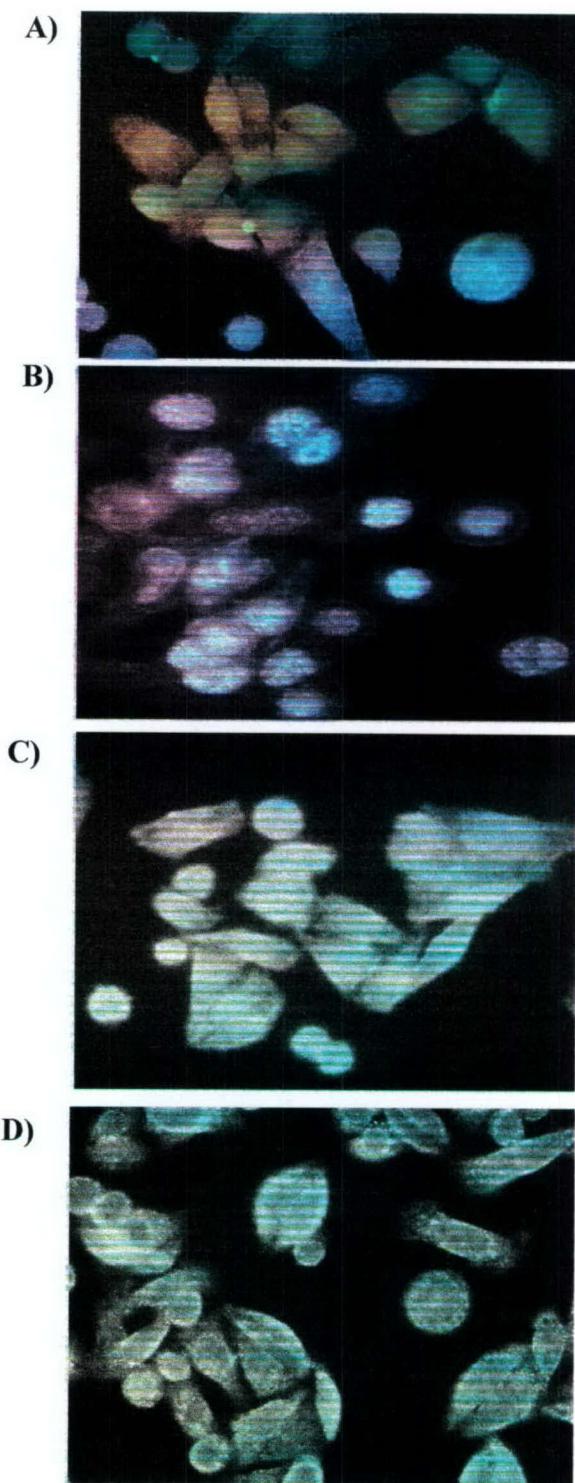
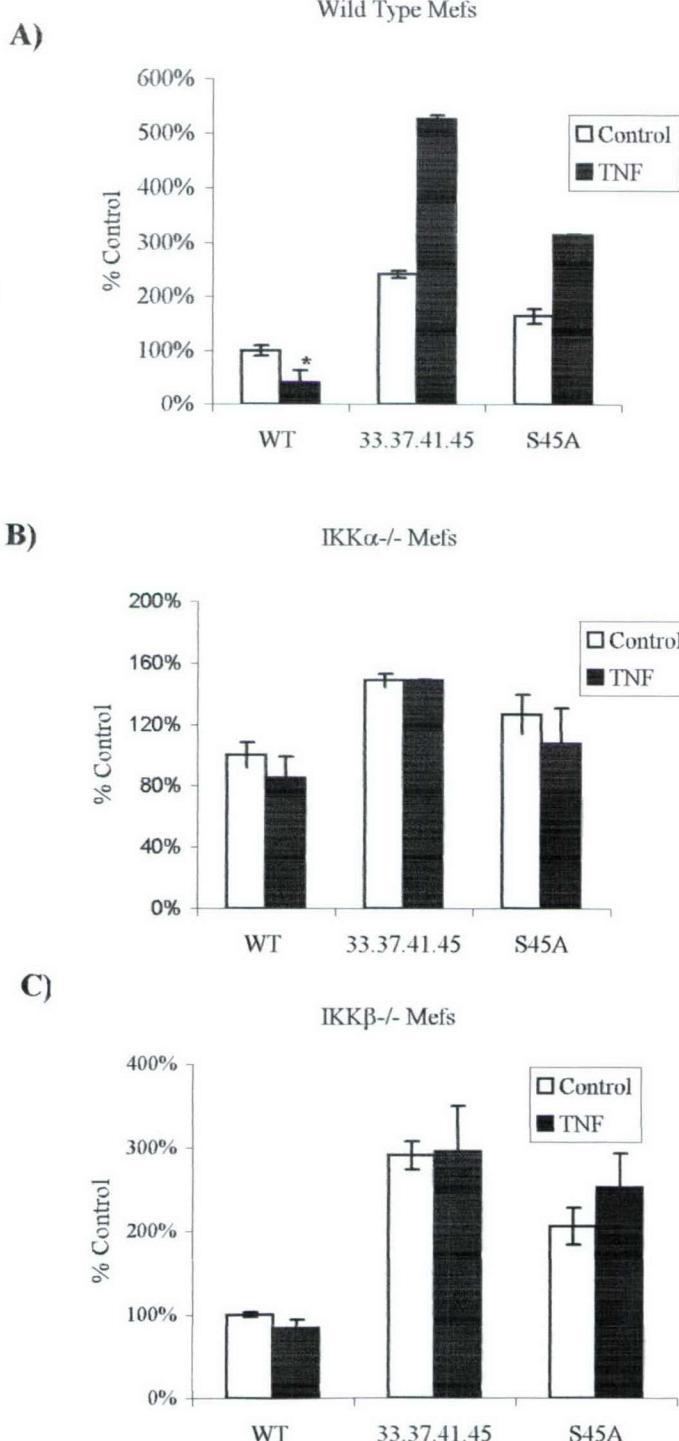


Figure 1.22 Effect of point mutants of b-catenin on TNFa repression of b-catenin signalling activity. A) Wild type mouse embryonic fibroblast cells (MEFs) were transfected with 0.5ug of wild type b-catenin, b-catenin mutated on residues 33,37,41,45 or b-catenin mutated on 45 alone for twenty-four hours. Cells were then treated with 20ng/ml of TNFa for further sixteen hours and harvested.

Luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ . B) Similar experiments were performed in IKK $\alpha$  (-/-) cells. C) Similar experiments were performed in IKK $\beta$  (-/-) cells.



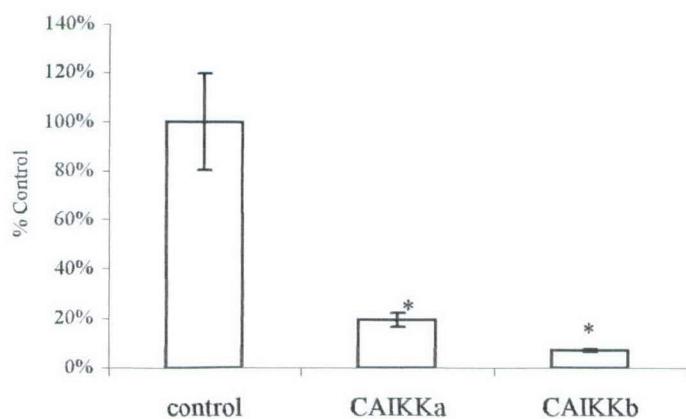


Figure 1.23: Effect of constitutively active (CA IKK) IKK mutants on b-catenin signaling activity. HCT 116 wt+/mut+ parental cells were transfected with 0.5ug of CA IKKa or IKKb, 0.1ug of Topflash reporter for twenty-four hours. Cells were harvested and luciferase activity was measured. Luciferase activity was measured. In all the reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

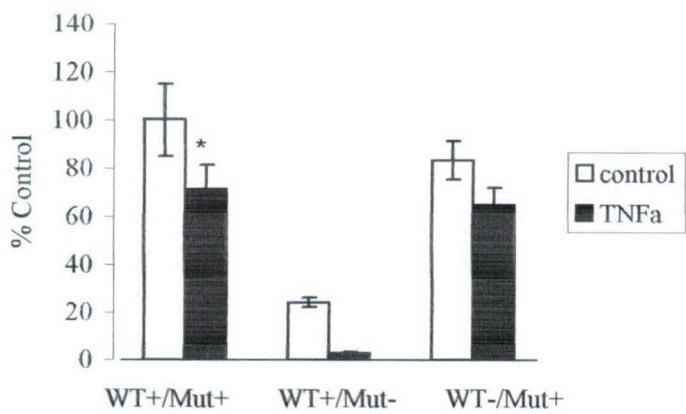


Figure 1.24 Effect of TNFa on b-catenin signaling in different forms of HCT-116 cell lines. Three different cell lines were used. WT+/Mut+ parental cells, and where either wild type (WT+/Mut+) (Mut) or mutant Ser45 allele (WT+/Mut-) (WT) was removed. These cell lines were transfected with 0.1ug of Topflash reporter and treated with 20ng/ml of TNFa over sixteen hours. Cells were harvested and luciferase activity was measured. In these reporter assays, experiments were performed in triplicates and repeated three times. Results were plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

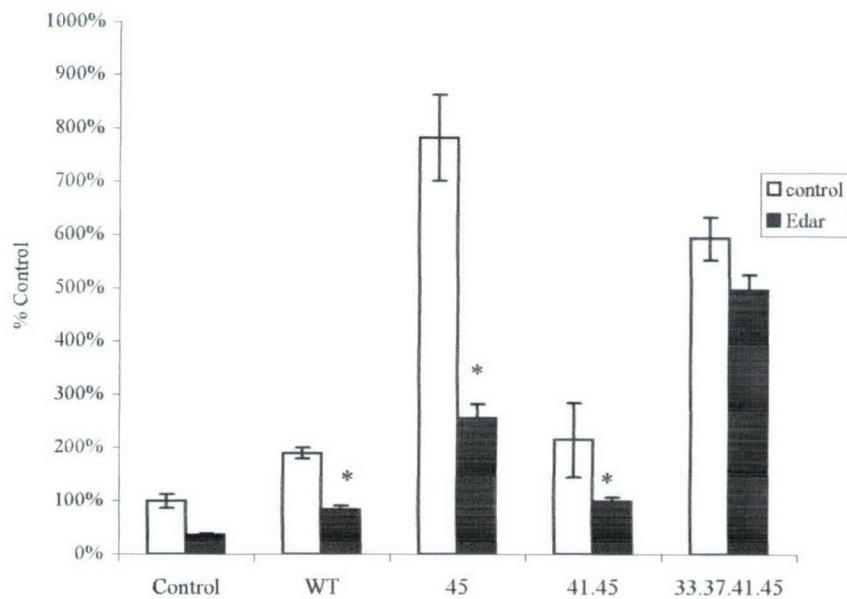


Figure 1.25 Effect of mutated  $\square$ -catenin on Edar regulation of  $\square$ -catenin signaling in wild type MEF cell lines. Wild Type mouse embryonic fibroblast cells (MEFs) were transfected with 0.5ug of wild type  $\square$ -catenin, or mutated  $\square$ -catenin at Ser45, Thr41, Ser45 and Ser33,Ser37,Thr41,Ser45, 50ng Edar and 0.1ug of Topflash reporter for twenty-four hours. In these reporter assays, experiments were performed in triplicates and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

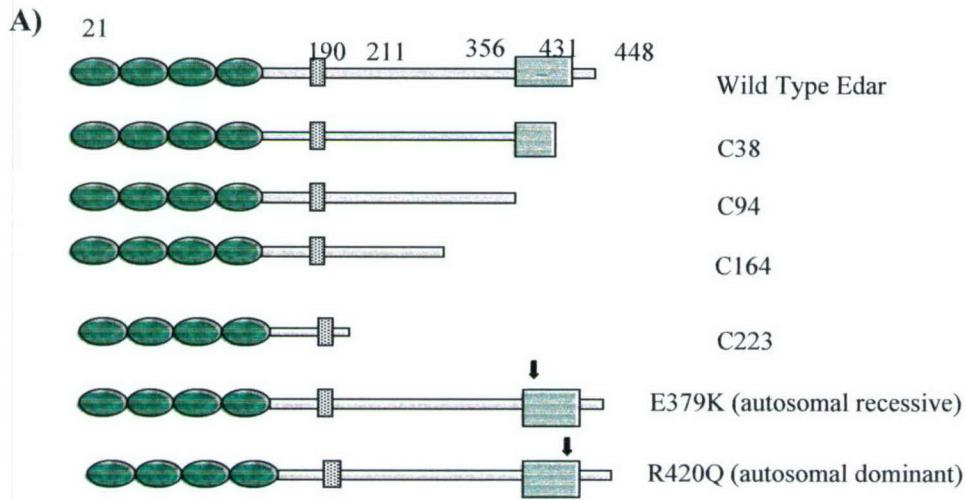
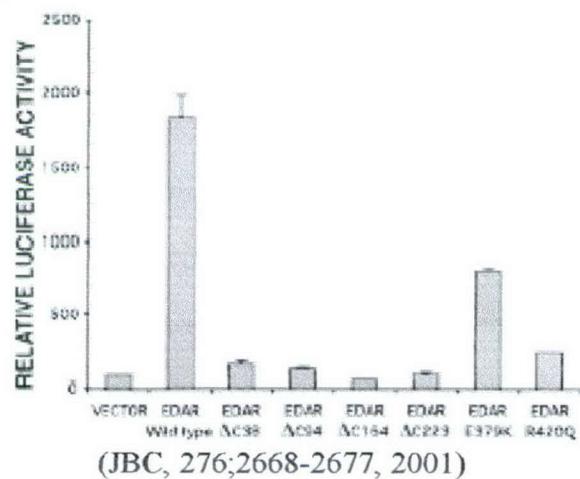


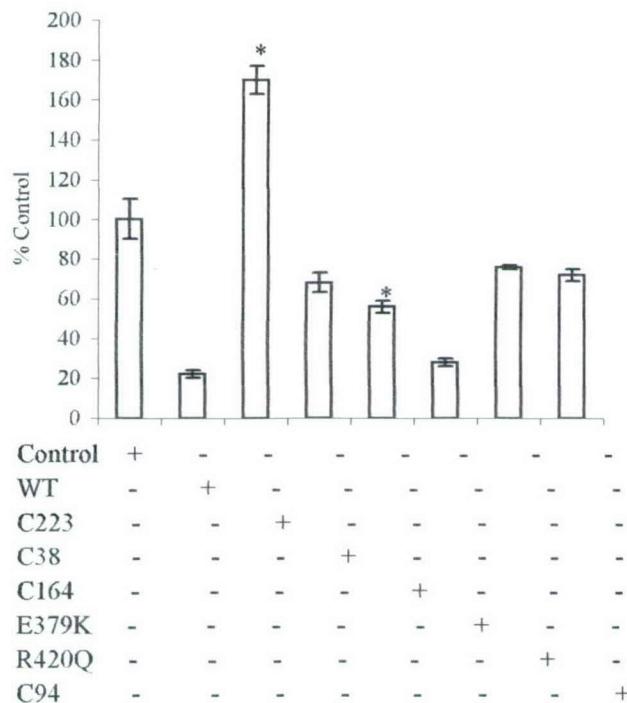
Figure 1.26 Involvement of the intracellular death domain of Edar in  $\beta$ -catenin signaling activity. A) Schematic representation of wild type Edar and its mutants. B) NFkB reporter assay reproduced from paper published by Chaudhary et al. (JBC, 276;2668-2677, 2001) HEK 293 cells were transfected with 0.5ug of wild type Edar or its mutants, along with an NFkB luciferase reporter construct (75 ng/well) and a Rous sarcoma virus promoter-driven  $\beta$ -galactosidase reporter construct (pRcRSV/LacZ; 75 ng) for twenty-four hours. Results were repeated three times and luciferase values were normalized with  $\beta$ -galactosidase activity to control for the difference in the transfection efficiency. C) Luciferase assay. SW480 cells were transfected with of 50ng of wild type Edar or its mutants and 0.1ug of Topflash reporter for twenty-four hours. In these reporter assays, experiments were performed in triplicate and repeated three times and luciferase values were normalized with renilla and plotted as % control. The average of three experiments,  $\pm$  standard deviation are plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

B)



(JBC, 276;2668-2677, 2001)

C)



## KEY RESEARCH ACCOMPLISHMENTS

- TNF alpha and Edar regulates beta-catenin signaling activity by repressing its signaling activity
- TNF alpha and IKK affect beta-catenin signaling independently of APC
- Constitutively active IKK alpha and IKK beta further down regulate beta-catenin signaling activity and augment NFkappaB signaling activity.
- Dominant negative IKK alpha and IKK beta reverse TNFalpha down-regulation of beta-catenin signaling activity and block NFkappaB signaling activity
- TNFalpha repression of beta-catenin signaling activity is abolished by IKK RNAi and in IKK -/- mouse embryonic fibroblasts cells
- Increase in NFkappaB signaling activity is not involved in TNFalpha repression of beta-catenin signaling activity
- The decrease in beta-catenin signaling activity is not due to a decrease in total beta-catenin protein levels, however, the dephosphorylated form of beta-catenin within the nucleus disappears after treatment with TNFalpha
- Transrepression of beta-catenin signaling activity involves the phosphorylation of N-terminal serine and threonine residues

## REPORTABLE OUTCOMES:

Manuscript submitted to MCB for publication. Awaiting outcome.

## CONCLUSION:

Initial data using dominant negative IKK mutants show that TNF alpha repression of beta-catenin is blocked when either IKK alpha or IKK beta is blocked. The use of knockout MEF cells further confirm this. We also confirmed that ectodysplasin utilizes a different pathway to regulate beta-catenin, of which we were unable to identify in this study. The most likely explanation for the differences in the role of the IKK complex in the TNF alpha and ectodysplasin pathways is that TNF alpha utilizes IKK heterodimers but not homodimers and ectodysplasin is only able to transducer signals through homodimers. Further studies would need to be done to elucidate the upstream mediators

involved in the ectodysplasin pathway in order to understand the role that it plays in epithelial development and how it regulates beta-catenin signaling.

REFERENCES:

1. **Jang MK, Goo YH, Sohn YC, Kim YS, Lee SK, Kang H, Cheong J, and Lee JW.** 2001. Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV stimulates nuclear factor-kappa B transactivation via phosphorylation of the p65 subunit. *J Biol Chem* 276: 20005-20010.
2. **Orsulic S, Huber O, Aberle H, Arnold S, and Kemler R.** 1999. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J Cell Sci* 112 ( Pt 8): 1237-1245.
3. **Rosin-Arbesfeld R, Townsley F, and Bienz M.** 2000. The APC tumour suppressor has a nuclear export function. *Nature* 406: 1009-1012.
4. **McMahon AP and Moon RT**, Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58: 1075-1084, 1989.
5. **Nagafuchi A and Takeichi M**, Cell binding function of E-cadherin is regulated by the cytoplasmic domain. *EMBO J* 7: 3679-3684, 1988.
6. **Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB, and Nathans J**, A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* 398: 431-436, 1999.

7. **Caca K, Kolligs FT, Ji X, Hayes M, Qian J, Yahanda A, Rimm DL, Costa J, and Fearon ER**, Beta- and gamma-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ.* 10: 369-376, 1999.
8. **Behrens J, Vakaet L, Friis R, Winterhager E, Van Roy F, Mareel MM, and Birchmeier W**, Loss of Epithelial Differentiation and Gain of Invasiveness Correlates with Tyrosine Phosphorylation of the E-Cadherin/B-Catenin Complex in Cells Transformed with a Temperature-Sensitive v-SRC Gene. *JCB* 120: 757-766, 1993.
9. **Easwaran V, Song V, Polakis P, and Byers S**, The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin- lymphocyte enhancer-binding factor signaling. *J.Biol.Chem.* 274: 16641-16645, 1999.
10. **Laney JD and Hochstrasser M**, Substrate targeting in the ubiquitin system. *Cell* 97: 427-430, 1999.
11. **Maniatis T**, A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev.* 13: 505-510, 1999.
12. **Winston JT, Strack P, Beer-Romero P, Chu CY, Elledge SJ, and Harper JW**, The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with

phosphorylated destruction motifs in IkappaBalphalpha and beta-catenin and stimulates IkappaBalphalpha ubiquitination in vitro [In Process Citation]. *Genes Dev.* 13: 270-283, 1999.

13. **Spiegelman VS, Slaga TJ, Pagano M, Minamoto T, Ronai Z, and Fuchs SY**, Wnt/Beta-catenin signaling induces the expression and activity of Beta-TrCP ubiquitin ligase receptor. *Molecular Cell* 5: 877-882, 2000.

14. **Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, and Woodgett JR**, Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation [In Process Citation]. *Nature* 2000.Jul.6.;406.(6791.):86.-90. 406: 86-90.

15. **Suzuki H, Chiba T, Kobayashi M, Takeuchi M, Suzuki T, Ichiyama A, Ikenoue T, Omata M, Furuichi K, and Tanaka K**, IkappaBalphalpha ubiquitination is catalyzed by an SCF-like complex containing Skp1, cullin-1, and two F-box/WD40-repeat proteins, betaTrCP1 and betaTrCP2. *Biochem.Biophys.Res Commun.* 256: 127-132, 1999.

16. **Lamberti C, Lin KM, Yamamoto Y, Verma U, Verma IM, Byers S, and Gaynor RB**, Regulation of beta-catenin function by the IkappaB kinases. *J Biol Chem* 276: 42276-42286, 2001.

17. **Nakamura T, Hamada F, Ishidate T, Anai K, Kawahara K, Toyoshima K, and Akiyama T**, Axin, an inhibitor of the Wnt signalling pathway, interacts with

beta- catenin, GSK-3beta and APC and reduces the beta-catenin level. *Genes Cells* 3: 395-403, 1998.

18. **Kawahara K, Morishita T, Nakamura T, Hamada F, Toyoshima K, and Akiyama T**, Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. *J Biol Chem* 275: 8369-8374, 2000.

19. **Staal FJ, Noort MM, Strous GJ, and Clevers HC**, Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. *EMBO Rep.* 3: 63-68, 2002.

#### Appendices

Manuscript submitted for publication

# **The IKK Complex Mediates TNF $\alpha$ Regulation of $\beta$ -catenin Signaling**

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Running Head: TNF $\alpha$  regulation of  $\beta$ -catenin signaling

Key Words:  $\beta$ -catenin, TNF, IKK, cyclin D1, APC.

## Abstract

The Wnt/β-catenin and TNF/IKB/NF-κB pathways are involved in cell cycle control, differentiation, and inflammation. Both IKB and β-catenin are regulated by phosphorylation at similar consensus serines and targeted for ubiquitination and degradation by the same ubiquitin ligase complex. The IKB kinase complex (IKK) that phosphorylates IKB contains two kinases, IKK $\alpha$  and IKK $\beta$ , which are activated in response to cytokines such as TNF $\alpha$ . We now show that TNF $\alpha$  inhibits β-catenin signaling activity independently of the tumor suppressor gene APC. In contrast, manipulation of APC/β-catenin signaling does not affect NF-κB activity. Using a variety of approaches, including dominant negative IKKs, RNAi and IKK knockout cells and animals we show that IKK $\alpha$  and IKK $\beta$  but not NF-κB mediate the effects of TNF $\alpha$  on β-catenin signaling activity. Although neither TNF $\alpha$  nor constitutively active IKKs reduce total β-catenin protein levels they markedly reduce the level of active de-phosphorylated β-catenin in the nucleus by targeting serine and threonine residues in the N-terminal regulatory domain of β-catenin. Levels of β-catenin and the β-catenin target gene cyclin D1 were elevated in the nuclei of epidermal cells in the abnormal skin of IKK $\alpha$  (-/-) mice. These data point to a role for cytokines and the IKK complex, in the normal regulation of β-catenin signaling activity.

## **Introduction:**

The Wnt/β-catenin and TNF/IκB/NF-κB pathway regulate a number of processes important in oncogenesis, development and the immune system. These processes include apoptosis, cell cycle control, adhesion and inflammation. In the absence of Wnt-signaling, β-catenin is phosphorylated sequentially by casein kinase 1 (CK1) and glycogen synthase kinase 3-β (GSK-3β) (4,52); activation of frizzled receptors by Wnt inhibits GSK-3β activity (52). Upon Wnt signaling, β-catenin accumulates in the cytoplasm and nucleus where it interacts with TCF/LEF transcription factor complexes to regulate the expression of genes including cyclin D1 and c-myc (9). Cytokines, such as TNFα stimulate the NF-κB signaling pathway, through the activation of many upstream kinases, including GSK-3β and the IκB kinases IKKα and IKKβ (14,15,25,66). In the absence of cytokines, IκB proteins inhibit NF-κB transcriptional activity by sequestering it in the cytoplasm (3). Activation of the IKK phosphorylates IκB resulting in its degradation and the release and nuclear translocation of NF-κB, which is then able to activate gene transcription (4,61). Both IκB and β-catenin are phosphorylated at similar consensus serines at the N-terminal and targeted for ubiquitination and degradation by the same ubiquitin ligase complex (2,2,29). β-catenin N-terminal phosphorylation also appears to regulate its transactivation properties independently of protein degradation (17). IKKα and IKKβ are components of a 600-900 kDa complex which also includes IKKγ/NEMO which has no catalytic activity but acts as a scaffold protein (34,38,49,65). Several studies have indicated that the Wnt and NF-κB pathways interact although it is not clear

CA) supplemented with 5% fetal bovine serum and 2mM L-glutamine. HEK 293 human embryonic kidney cells were also grown in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum and 2mM L-glutamine. IKK $\alpha$ (-/-) and IKK $\beta$ (-/-) mouse embryo fibroblasts (MEF) cell lines were grown in Dulbecco's modified Eagle's medium and supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% penicillin/streptomycin (29).

**Plasmid Constructs and Transfections:** SW480 and HEK 293 cells were transfected according to the manufacturer's recommendations using FuGENE 6 Reagent (Roche Diagnostics). IKK $\alpha$ (-/-) and IKK $\beta$ (-/-) mouse embryonic fibroblast cells were transfected using Lipofectamine Plus (Invitrogen, CA) according to the manufacturer's recommendations. F. Mercurio kindly provided the IKK $\alpha$  (S176/180E, also known as constitutively active, CA IKK $\alpha$  and S176/180A, also known as dominant negative, DN IKK $\alpha$ ) and IKK $\beta$  (S177/181E, also known as constitutively active, CA IKK $\beta$  and S177/181A, also known as dominant negative, DN IKK $\beta$ ) and IKK $\alpha$  (K44M) and IKK $\beta$  (K44M) mutants (39,63). B. Vogelstein kindly provided the APC plasmid, W. Li provided the dominant negative mutants to PI3 kinase and AKT, C. Duckett provided K63W TAK1 and TAB1, and siamois reporter plasmid was provided by D. Kimelman.

Deletion constructs of  $\beta$ -catenin were made via PCR using primers of the following:

$\Delta N$ : forward primer 5'- CGGGATCCATGCGTGCAATCCCTGAAGT -3' reverse primer 5'- GCTCTAGATCATTACAGGTCAGTATCAAACCAGGCC-3'  $\Delta C$ : forward primer 5'- CGGGATCCATGGCTACTCAAGCTGATTG -3' reverse primer 5'-

GGCTCTAGAGCCTATGCGTAGTCCGGCACGTCGTACGGTAAGAGCTGGTC  
AGCTC -3' The inserts were digested using restriction enzymes, BamH1 and Xba1. They  
were ligated into pcDNA3 vector (Invitrogen), transformed and selected with ampicillin.

**Site Directed Mutagenesis:** Mutations were performed using Pfu proofreading polymerase and QuikChange<sup>TM</sup> XL Site-Directed Mutagenesis Kit (Stratagene) with modifications made to the PCR cycling temperatures. The following primers were used:  
T41A: 5'- CTGGTGCCACTGCCACAGCTCCTCTCTG -3'.  
S45A: 5'- CCACAGCTCCTGCTCTGAGTGGTAAAGGC -3'  
S33A, S37A: 5'- CAGTCTTACCTGGACGCTGGAATCCATGCT-3' S33A, S37A  
mutant was made using the S37A template in pcDNA3 vector (Invitrogen)  
S33A, S37A, T41A, S45A: 5'- TACCTGGACGCTGGAATCCATGCTGGTGCC -3'  
S33A, S37A, S45A, T41A mutant was made using the S33A, S41A, S45A mutant  
template.

**TCF/LEF Reporter Assay:** Cells were plated at 100,000 cells/well and experiments were performed in 12 well cell culture plates. 24 hours after plating, the cells were transfected with the indicated DNA and one of the reporters; TOPFlash (0.1ug), FOPFlash (0.1ug) or NF- $\kappa$ B luciferase (0.1ug). Dose responses of dominant negative (DN IKK $\alpha$  and DN IKK $\beta$ ) ranged from 0.05-1.0 ug. 0.2ug of  $\beta$ -catenin and their point mutants were used to transfect MEFs. Renilla luciferase reporter (1ng) was used as an internal control to correct for transfection efficiency. All transfections were done in triplicate and repeated at least 3 times with the TCF/LEF reporter activity measured in

lumens after 16 hours of TNF $\alpha$  treatment or 24 hours after transfections using Dual Luciferase Reporter Assay System kit (Promega). Dose responses were also performed and optimal doses were chosen for these experiments.

**RNA oligonucleotides:** siRNAs with two thymidine residues (dTdT) at the 3' end of the sequence were designed for IKK $\alpha$  (sense 5'-AAGCAGGCTTTCAAGGGACACCTGTCTC -3'), IKK $\beta$  (sense 5'-AAGTCGACGGTCACTGTGTACCCGTCTC -3'), p65 (sense 5'-AAGCCCTATCCCTTACGTACACCTGTCTC -3'), non-specific RNA oligonucleotides (sense 5'-AAGAGGGGGTTCCATTAAGCCCTGTCTC -3') genes along with their corresponding antisense RNA oligonucleotides as described. (\*) These RNAs were dissolved in RNase free water as 100 $\mu$ M solutions. RNA oligonucleotides targeting cadherin

**Transfections of RNA oligonucleotides:** Approximately, 1 X 10<sup>6</sup> cells were plated per 12-well plate in containing 5% FBS to give 40-50% confluency and transfection of the RNA oligonucleotides was performed using Oligofectamine (Invitrogen, Carlsbad, CA) to result in a final RNA concentration of 25nM for IKK $\alpha$  and 100nM for IKK $\beta$  and p65. The cells were transfected consecutively with the oligonucleotides for 2 days and they were harvested 48 hours post-transfection with TOPFlash and NF- $\kappa$ B reporters. They were treated with TNF $\alpha$  24 hours post-transfection.

**Western Blotting:** Cells were grown to confluence in 100mm dishes (48 hours after transfection) and washed twice with phosphate buffered saline (PBS). They were lysed for 10 minutes on ice in NP-40 lysis buffer (1% NP-40 and protease inhibitors including 1mM sodium vanadate, 50mM sodium fluoride and Boerhringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet). Lysates were centrifuged at 14,000 rpm at 4°C. For pure cytoplasmic lysates, cells were washed with PBS as described and lysed for 10 minutes on ice in Swell Buffer (10mM Tris pH7.5, 0.2mM magnesium chloride). Cells were scraped and dounced, after which, inhibitor cocktail (1.25M sucrose, 250mM sodium fluoride, 5mM EDTA, 5mM sodium vanadate and Boerhringer Mannheim complete mini EDTA-free protease inhibitor cocktail tablet) was added. Lysates were centrifuged at 28,800 rpm at 4°C for 1 hour. The supernatant was mixed with ethanol and left overnight at -20°C. This was then centrifuged at 28,800 rpm at 4°C for 30 minutes. The precipitate was dissolved in sample buffer (2% sodium dodecyl sulphate, 60mM Tris, pH6.8, 10% glycerol). Protein content was measured by BCA protein assay (Pierce Laboratories). The procedure was performed as previously described (41). 3-8% tris-acetate NuPage gels (Invitrogen Corporation) were used for SDS-PAGE. Monoclonal antibody to  $\beta$ -catenin was obtained from Transduction Laboratories. Monoclonal antibody against HA tag (Boerhinger Mannheim), monoclonal FLAG antibody (Eastman Kodak) and monoclonal antibodies against IKK $\alpha$  and IKK $\beta$  and de-phosphorylated  $\beta$ -catenin (Upstate Biotechnology) were also used in Western blot analysis and immunocytochemistry. The blots were developed using ECL chemiluminescent detection (Amersham Pharmacia Biotech). Some blots were stripped (62.5mM Tris pH 7.5, 2% SDS, 1.7%  $\beta$ -mercaptoethanol) for 25 minutes at 50°C, re-

blocked with 5% non-fat milk and re-probed with new primary and secondary antibody according to manufacturer's recommendations.

**Immunocytochemistry:** Cells were grown to confluence in 175 cm<sup>2</sup> flasks and trypsinized using trypsin (Invitrogen Corporation) where 150,000 cells per well were plated on 18mm cover slips in 12 well plates. The cells were transfected as described above with 1ug of HA-tagged CA IKK $\alpha$  or Flag-tagged CA IKK $\beta$ . After 48 hours, they were washed twice with phosphate buffered saline (PBS) and fixed in 2% paraformaldehyde at room temperature for 30 minutes and permeabilized in 0.2% TritonX-100 at room temperature for 5 minutes. With TNF $\alpha$  treatment, the cells were treated with it for 16 hours and similar procedures were carried out. Purified rabbit HA antibody (Zymed Laboratories) or Flag antibody was used (Sigma-Aldrich) and normal IgG fluorescein or Texas Red® dye-conjugated (Kirkegaard and Perry Laboratories) were used as secondary antibodies for staining. All primary antibodies were incubated overnight at 4°C and all secondary antibodies were used at a 1:500 dilution at room temperature unless otherwise noted above. Imaging was done using a Nikon E600 Flourescence Microscope System with FITC or TRITC filter.

**Immunohistochemistry:** YinLing Hu and Michael Karin kindly provided the IKK $\alpha$  knockout and wild-type mouse paraffin blocks (22). Paraffin sections were dewaxed in xylene and rehydrated sequentially with 100, 95 and 50% ethanol in water. Sections were boiled in a solution of 10mM sodium citrate, pH 6.0 and microwaved on high power for 3x3 minute cycles, with 1 minute between cycles. They were allowed to cool for 30

minutes, rinsed 3 times in PBS and incubated in 0.3% peroxide solution at 4°C for 30 minutes. They were rinsed in PBS and air dried, after which the samples were circled with a PAP pen. The following antibodies were used: mouse IgG (Zymed Laboratories) and monoclonal  $\beta$ -catenin (Transduction Laboratories). Sections were incubated in horse anti-mouse biotinylated secondary antibody (Vector Laboratories) at room temperature for 60 minutes. The AEC Substrate Kit (Zymed Laboratories) was used to detect staining and some sections were lightly counterstained with hematoxylin. Cyclin D1 immunocytochemistry was also carried out as described earlier.(36)

## Results:

**TNF $\alpha$  and IKK affect  $\beta$ -catenin signaling independently of APC:** In previous studies, we reported that treatment of  $\beta$ -catenin-transfected HeLa cells with TNF $\alpha$  activated the IKK complex and increased the phosphorylation of  $\beta$ -catenin (29). To further understand how TNF $\alpha$  regulates  $\beta$ -catenin, we studied its effect on  $\beta$ -catenin signaling in SW480 colon cancer cells. These cells have a truncated APC gene and thus have high endogenous  $\beta$ -catenin protein levels and signaling. To measure  $\beta$ -catenin signaling, we used a luciferase promoter containing TCF/LEF response elements (TOPFLASH) (58). SW480 cells express the TNF $\alpha$  receptor and treatment with the ligand results in apoptosis after 48h (19,24). Treatment of SW480 cells with 20ng/ml TNF $\alpha$  for 16 hours significantly increased NF- $\kappa$ B reporter activity and decreased  $\beta$ -catenin reporter activity (**Fig. 1A**). Similar results were obtained in 293 cells, which have lower (but still detectable) endogenous levels of  $\beta$ -catenin signaling and express wild-type APC (**Fig. 1B**). TNF $\alpha$  also inhibited  $\beta$ -catenin/TCF signaling in 293 cells transfected with  $\beta$ -catenin (not shown) as well as the activity of the  $\beta$ -catenin/TCF sensitive siamois reporter in a similar manner (**Fig. 1C**). The siamois reporter contains TCF/LEF sites as well several other potential transcription factor binding sites (6) However, the effects of TNF $\alpha$  on siamois promoter activity are mediated by the interaction of  $\beta$ -catenin with TCF/LEF sites since TNF $\alpha$  had little effect on the activity of a siamois promoter in which the TCF sites were mutated. No significant changes in the activity of a renilla-luciferase control reporter occurred and we did not observe any cell death after the 16 hrs treatment period. Thus our data show that the effect of TNF $\alpha$  on  $\beta$ -catenin signaling is independent of APC

(it works both in the absence (SW480 cells) and presence (293 cells) of APC) but does depend on TCF binding elements.

TNF $\alpha$  exerts its effects on NF- $\kappa$ B by activating the IKK complex (12,67). NF- $\kappa$ B-inducing kinase (NIK) can also activate the IKK complex but does so independently of TNF $\alpha$ . We next wanted to test the hypothesis that activation of IKK, either by TNF $\alpha$  or NIK could regulate  $\beta$ -catenin signaling. Like TNF $\alpha$ , expression of NIK decreased  $\beta$ -catenin signaling and increased NF- $\kappa$ B activity (**Fig. 1D**). To further relate the activation of the IKK complex with  $\beta$ -catenin signaling, we transfected SW480 cells with constitutively active IKK mutants and TOPFlash. The constitutively active mutants (CA IKK $\alpha$  and CA IKK $\beta$ ) were generated by changing serines residues located within the highly conserved activation loops at serines 176 and 180 in IKK $\alpha$  and 177 and 181 in IKK $\beta$  to glutamate (SS to EE) (39). This mimics the phosphorylation induced conformational change to result in kinase activation.  $\beta$ -catenin signaling decreased by 80-90% upon transfection of either CA IKK $\alpha$  or CA IKK $\beta$ . (**Fig. 1E**) The effects of CA IKK $\alpha$  and CA IKK $\beta$  on  $\beta$ -catenin and NF- $\kappa$ B activity were further augmented by treatment of cells with TNF $\alpha$  (**Fig. 1E**). Kinase inactive mutants of IKK $\alpha$  and IKK $\beta$  did not inhibit  $\beta$ -catenin signaling activity (**Fig. 1F**). Similar results were obtained using the cyclin D1 promoter (**Fig. 1G**). Because NF- $\kappa$ B positively regulates cyclin D1 promoter activity, these data indicates that in APC-mutant colon cancer cells, TNF $\alpha$  regulation of the -163 cyclin D1 promoter is predominantly regulated by the  $\beta$ -catenin/TCF and not

the NF-κB site (54). Consistent with this, the activity of a cyclin D1 promoter construct with a mutated TCF site was not inhibited by CA IKKs.

**The IKK complex mediates the effects of TNF $\alpha$  but not APC on  $\beta$ -catenin signaling:**

In order to identify the relative contribution of IKK $\alpha$  and IKK $\beta$  in the TNF $\alpha$ -induced down-regulation of  $\beta$ -catenin reporter activity, we transfected SW480 cells with dominant negative IKK $\alpha$  (S176/180A) or dominant negative IKK $\beta$  (S177/181A) (Fig. 2A). The alanine substitutions for serine residues in the activation loops of these kinases inhibit kinase activation by upstream mediators. Both dominant negative mutants effectively blocked the down-regulation of TOPFlash and induction of NF-κB reporter by TNF $\alpha$ . Like TNF $\alpha$ , expression of wild-type APC in APC-mutant SW480 cells also inhibits  $\beta$ -catenin signaling. Although  $\beta$ -catenin signaling was reduced dramatically by APC, NF-κB reporter activity was completely unaffected and DN IKKs did not affect the ability of APC to inhibit  $\beta$ -catenin signaling activity (Fig. 2B). In addition, APC did not potentiate or inhibit the ability of TNF $\alpha$  to regulate NF-κB activity in SW480 cells.

We also used RNAi to inhibit IKK $\alpha$  and IKK $\beta$  expression. Western blot analysis showed a marked loss of IKK protein following RNAi treatment (not shown). Repression of  $\beta$ -catenin signaling by TNF $\alpha$  was markedly inhibited in the presence of IKK $\alpha$  RNAi (Fig. 2C). Similar results were obtained with IKK $\beta$  RNAi but large amounts of RNAi were necessary to reduce endogenous IKK $\beta$  levels. Even though our results are related to non-specific RNAi, we were concerned that the large amount of IKK $\beta$  RNAi that was used in

these experiments may have adverse non-specific effects on the cell. To confirm the role of IKK $\beta$  and IKK $\alpha$  in TNF $\alpha$  regulation of  $\beta$ -catenin signaling, we used mouse embryonic fibroblasts (MEFs), which were disrupted in IKK $\alpha$  or IKK $\beta$  and performed similar experiments (23,35). When either IKK $\alpha$ (-/-) or IKK $\beta$ (-/-) or MEFs were used, TNF $\alpha$  was unable to down-regulate  $\beta$ -catenin signaling activity (**Fig.2C**). NF $\kappa$ B signaling in response to TNF $\alpha$  was also significantly inhibited in the IKK $\alpha$  (-/-) and IKK $\beta$  (-/-) MEFs. Taken together these data show unequivocally that IKK $\alpha$  and IKK $\beta$  are involved in TNF $\alpha$  regulation of  $\beta$ -catenin signaling activity. Although NIK can activate the IKK complex and decrease  $\beta$ -catenin signaling (**Fig. 1D**), dominant negative NIK could not reverse the effects of TNF $\alpha$  on TOPFlash or NF- $\kappa$ B activity, consistent with previous data showing that NIK is not required for TNF $\alpha$  activation of IKK (50) (**Fig. 2E**). Although it does not activate NIK, TNF $\alpha$  can activate a number of kinases other than IKK, including, PI3 kinase, Akt, and importantly, GSK-3 $\beta$  (7,21,43,53). We next tested the role of these kinases by transfecting dominant negative PI3 kinase, Akt, and by treating cells with LiCl to inhibit GSK-3 $\beta$ . None of these manipulations affected the ability of TNF $\alpha$  to inhibit  $\beta$ -catenin signaling further pointing to the important role of the IKK complex in mediating the effects of TNF $\alpha$  on  $\beta$ -catenin signaling (**Fig. 2E**).

**TNF $\alpha$  repression of  $\beta$ -catenin activity is independent of NF $\kappa$ B signaling activity:** We showed previously that IKK effects on  $\beta$ -catenin signaling in  $\beta$ -catenin transfected COS cells were unaffected by inhibition of NF- $\kappa$ B activation by mutated I $\kappa$ B (29). We also found that mutated I $\kappa$ B did not influence the effects of CA IKK mutants on  $\beta$ -catenin

signaling in SW480 cells even though it completely inhibited the activation of NF- $\kappa$ B (not shown). This indicates that inhibition of NF- $\kappa$ B activation downstream of IKK does not affect  $\beta$ -catenin signaling. However, there is some evidence that exogenously over-expressed NF- $\kappa$ B may play a role in the regulation of  $\beta$ -catenin signaling activity by suppressing  $\beta$ -catenin dependent transcription (11,37). We wanted to confirm if TNF $\alpha$  mediated repression of  $\beta$ -catenin signaling is indeed independent of TNF $\alpha$  activation of NF- $\kappa$ B. Of the five NF- $\kappa$ B subunits in mammalian cells, RelA (p65), RelB and c-Rel contain transactivation domains in the C-terminus and require no additional processing to their active forms (62) We found that SW480 cells and MEFs express p65 and used siRNA to silence it. Our western analysis confirmed that p65 was silenced following treatment (**Fig. 3A**) and that loss of p65 prevented the activation of the NF $\kappa$ B reporter by TNF $\alpha$  (**Fig.3B**). However, even in the absence of p65 protein TNF $\alpha$  continued to repress  $\beta$ -catenin signaling activity (**Fig.3C**). This data strongly suggests that TNF $\alpha$  regulates  $\beta$ -catenin signaling activity independently of NF- $\kappa$ B.

**TNF $\alpha$  and activated IKK regulate the localization of activated, de-phosphorylated  $\beta$ -catenin:** Earlier studies showed that TNF $\alpha$  and CAIKK mutants resulted in phosphorylation of exogenously expressed  $\beta$ -catenin (29). We showed several years ago that phosphorylation of certain serine residues in the N-terminal of  $\beta$ -catenin can regulate its stability by targeting it for ubiquitination (29) Although a putative IKK consensus sequence is present in the N-terminal region of  $\beta$ -catenin and is a likely target for IKK, mapping studies indicated that several other regions of  $\beta$ -catenin can serve as substrates

for IKK (29). It is also possible that IKK may affect  $\beta$ -catenin mediated trans-activation by phosphorylating another component of the transcriptional machinery. If  $\text{TNF}\alpha$  or activation of IKK regulates  $\beta$ -catenin signaling by targeting it for ubiquitination and protein degradation, one would expect that like APC, axin or activated GSK-3 $\beta$  they would affect  $\beta$ -catenin protein levels (26,40). However, treatment with  $\text{TNF}\alpha$  did not alter cytoplasmic protein levels of total  $\beta$ -catenin in SW480 cells as measured by western blotting with an antibody directed at the C-terminal (Fig. 4A). In 293 cells, which have much less cytoplasmic  $\beta$ -catenin than SW480 cells treatment with  $\text{TNF}\alpha$  actually appeared to increase  $\beta$ -catenin protein levels (Fig. 4B). Immunocytochemical studies showed that treatment with  $\text{TNF}\alpha$  had no obvious effect on  $\beta$ -catenin protein localization (results not shown). Similarly, double-labeling studies of  $\beta$ -catenin in SW480 cells transiently transfected with CA IKKs revealed no significant alteration in  $\beta$ -catenin levels or localization in the cells expressing IKKs (Fig. 5A-C). This is in marked contrast to SW480 cells transfected with APC or axin/conductin in which addition of these components of the  $\beta$ -catenin degradation pathway results in a marked loss of nuclear and cytoplasmic  $\beta$ -catenin concomitant with changes in TOPFlash activity (18,20). Some studies have shown that phosphorylation of  $\beta$ -catenin on serines 33, 37, 45 and threonine 41 regulates its cellular localization and transcriptional activation functions, in addition to or instead of regulating protein degradation (17) (17,51). In addition, IKKs directly affect the transcriptional activity of NF- $\kappa$ B as well as targeting I $\kappa$ B for degradation (46,64). Recently, reagents that specifically recognize phosphorylated (inactive) or dephosphorylated (active) forms of  $\beta$ -catenin have been developed (55). In untreated

SW480 cells activated  $\beta$ -catenin was localized to the nucleus and occasionally at cell-cell contact sites. Remarkably and in contrast to total  $\beta$ -catenin, activated  $\beta$ -catenin was almost always absent from the nuclei of cells transiently transfected with CA IKK $\alpha$  or  $\beta$  (Fig. 5D-F). No change in the localization of activated  $\beta$ -catenin was observed in cells expressing DN IKKs (not shown). These data are quantitated in figure 5G-J. Similar results were obtained in SW480 cells treated with TNF $\alpha$ , in which activated  $\beta$ -catenin was significantly reduced in the nucleus. (Fig. 6A-B) These data are quantitated in Fig. 6G. Note that this quantitation does not take into account cells with reduced but still detectable nuclear localization of activated  $\beta$ -catenin after treatment with TNF $\alpha$  and therefore represent an underestimate of the effects of TNF $\alpha$ . These data indicate that the effects of TNF $\alpha$  on  $\beta$ -catenin signaling are not associated with increased degradation of total  $\beta$ -catenin but do involve changes in the localization/level of activated  $\beta$ -catenin.

**TNF $\alpha$  trans-repression of  $\beta$ -catenin activity involves phosphorylation of N-terminal serine and threonine residues:**

We wanted to investigate if phosphorylation sites within the N-terminus might be involved in  $\beta$ -catenin regulation by TNF $\alpha$ . SW480 cells were stained with antibodies that recognize  $\beta$ -catenin only when it is phosphorylated on serines 41 and 45 (21). Little staining was observed in control cells but a dramatic increase in nuclear staining was observed 5-15 minutes following treatment with TNF $\alpha$  (Fig. 6C-D). Treatment of SW480 cells with TNF $\alpha$  also resulted in a change in staining using an antibody specific for  $\beta$ -catenin when it is phosphorylated on serines 33, 37 and threonine 41. This staining

pattern was cytoplasmic/membrane rather than nuclear. (**Fig 6E-F**) Similar results were observed in HEK293 cells (not shown). These data indicate that one or more of the N-terminal serine residues are important for TNF $\alpha$  to trans-repress  $\beta$ -catenin activity. To formally test this hypothesis we transfected mouse embryonic fibroblast cells (MEFs) with  $\beta$ -catenin,  $\Delta N\beta$ -catenin or  $\beta$ -catenin mutated either, on residues 33,37,41,45 or on serine 45 alone. Expression of  $\beta$ -catenin in wild-type MEFs increased  $\beta$ -catenin signaling and treatment of transfected cells with TNF $\alpha$  decreased this exogenous signaling activity (**Fig 7A**). In contrast, wild type (wt) MEFs transfected with  $\Delta N\beta$ -catenin,  $\beta$ -catenin mutated on residues 33,37,41,45 or on serine 45 alone were resistant to repression by TNF $\alpha$ . Interestingly, TNF $\alpha$  consistently increased the already elevated activity of  $\beta$ -catenin mutated on residues 33,37,41,45 or on serine 45. As expected IKK $^{-/-}$  MEFs transfected with either wt  $\beta$ -catenin or the various  $\beta$ -catenin mutants were insensitive to repression by TNF $\alpha$  (**Fig. 7B-C**). Remarkably, the ability of TNF $\alpha$  to increase the activity of mutated forms of  $\beta$ -catenin observed in wild type cells was also abolished in both IKK $\alpha$ ( $^{-/-}$ ) and IKK $\beta$ ( $^{-/-}$ ) cells.

These data also suggest that serine 45 is an important residue in the regulation of  $\beta$ -catenin repression by TNF $\alpha$  and IKK. To specifically test this we used another colon cancer cell line, HCT116, which has normal APC but expresses one allele of  $\beta$ -catenin mutated on serine 45 and one normal allele. We used parental HCT116 cells and HCT116 cells in which either the normal or the mutated  $\beta$ -catenin allele has been removed by somatic cell knockout (27). Remarkably, cells in which the normal allele had been removed (only expressing mutant  $\beta$ -catenin) were resistant to the effects of TNF $\alpha$

whereas cells in which the mutant allele was deleted (only expressing normal  $\beta$ -catenin) were highly sensitive and the parental cell line only moderately sensitive (**Fig. 7D**). These data are consistent with the recent demonstration that serine 45 is a site for IKK phosphorylation (47).

**$\beta$ -catenin and cyclin D1 are localized in the nuclei of epidermal cells in the neonatal IKK $\alpha$ (-/-) mouse**

Our results indicate a role for the IKK complex in the regulation of  $\beta$ -catenin localization and signaling. A number of studies indicate that IKK $\alpha$  does not play a dominant role in the regulation of NF- $\kappa$ B signaling (10,33,35). Although the IKK $\alpha$  (-/-) mouse does not have a marked NF $\kappa$ B phenotype, it does exhibit a thickening of the epidermis and has a defect in keratinocyte differentiation (22,33,56). Moreover, Hu et. al. had shown that the role of IKK $\alpha$  in keratinocyte differentiation is not exerted through IKK activation of NF- $\kappa$ B (23). Because elevated  $\beta$ -catenin signaling is associated with hyper-proliferation of epidermal basal cells, and the wnt/ $\beta$ -catenin/TCF pathway is implicated in epithelial stem cell proliferation and differentiation, we investigated  $\beta$ -catenin localization in the epidermis of IKK $\alpha$  (-/-) mice. Unfortunately, we were unable to use the antibody to activated  $\beta$ -catenin on paraffin sections but were able to use an antibody directed against the C-terminal of  $\beta$ -catenin. Membrane  $\beta$ -catenin staining was observed in both wild-type and IKK $\alpha$  (-/-) epidermis but a marked increase in the number of cells with nuclear  $\beta$ -catenin occurred throughout the epidermis and particularly in the basal region of IKK $\alpha$  (-/-) mice (**Fig. 8A**). Very few cells in the normal epidermis exhibited nuclear  $\beta$ -catenin

staining at this stage in development. Nuclear  $\beta$ -catenin staining also occurs in human epidermal keratinocytes expressing N-terminal deleted form of  $\beta$ -catenin but not in normal keratinocytes or in normal human skin (68). Localization of  $\beta$ -catenin in the nucleus suggests an increase in  $\beta$ -catenin/TCF signaling. Thus we localized the  $\beta$ -catenin target gene cyclin D1. Like  $\beta$ -catenin, cyclin D1 was markedly increased in the basal regions of the IKK $\alpha$  (-/-) mouse epidermis. (**Fig. 8G,H**)

## **Discussion:**

Cells produce a wide array of cytokines, which are important for the generation of inflammatory and immune responses. TNF $\alpha$  and other members of the TNF family also play a role in a variety of oncogenic and developmental processes and there are several indications of interaction between TNF/IKK/NF- $\kappa$ B and wnt/GSK-3/ $\beta$ -catenin pathways. For example, the TNF $\alpha$ -activated kinases IKK $\alpha$  and IKK $\beta$  can interact with and phosphorylate  $\beta$ -catenin (this study and (29)). GSK-3 $\beta$  and NF $\kappa$ B also appear to exert direct regulation over both pathways (11,21). In the present study we show that IKK $\alpha$  and IKK $\beta$  but not NF- $\kappa$ B mediate the effects of TNF $\alpha$  on  $\beta$ -catenin signaling activity. In addition  $\beta$ -catenin and the  $\beta$ -catenin target gene cyclin D1 accumulated in the nuclei of epidermal cells in the abnormal skin of IKK $\alpha$ (-/-) mice. These data, together with other published studies point to a role for cytokines and the IKK complex in the normal regulation of  $\beta$ -catenin signaling activity.

### **TNF $\alpha$ repression of $\beta$ -catenin activity is independent of NF- $\kappa$ B signaling activity:**

Two studies have shown that exogenously over-expressed NF- $\kappa$ B may play a role in the regulation of  $\beta$ -catenin signaling activity by directly interacting with it (11,37). We wanted to confirm if TNF $\alpha$  mediated repression of  $\beta$ -catenin signaling is indeed independent of TNF $\alpha$  activation of NF $\kappa$ B. Using RNAi to deplete p65 we showed that even in the absence of p65 protein TNF $\alpha$  continued to repress  $\beta$ -catenin signaling activity. This data demonstrates that TNF $\alpha$  regulates  $\beta$ -catenin signaling activity

independently of NF- $\kappa$ B. Consistent with this we showed previously that IKK effects on  $\beta$ -catenin signaling in  $\beta$ -catenin transfected COS cells were unaffected by inhibition of NF- $\kappa$ B activation by mutated I $\kappa$ B (25). This indicates that NF- $\kappa$ B activation downstream of IKK does not mediate TNF $\alpha$  repression of  $\beta$ -catenin signaling. However, these data do not exclude a role for direct  $\beta$ -catenin/NF- $\kappa$ B interactions in the regulation of promoters containing both TCF/LEF and NF- $\kappa$ B sites. Indeed in other studies we show that the  $\beta$ -catenin/TCF sensitive FGF-BP and cyclin D1 promoters are co-ordinately regulated by both NF- $\kappa$ B and  $\beta$ -catenin/TCF sites (48 and unpublished results).

**Inhibition of either IKK $\alpha$  or IKK $\beta$  prevents TNF $\alpha$ -mediated repression of  $\beta$ -catenin signaling:**

Experiments utilizing dominant negative IKKs, siRNA and IKK knockout cells showed definitively that the ability of TNF $\alpha$  to repress  $\beta$ -catenin signaling could be blocked if either IKK $\alpha$  or IKK $\beta$  was missing or inhibited. Remarkably, although IKK $\alpha$  is not required for TNF activation of NF- $\kappa$ B in some cells, it is essential for the TNF-related ligand, RANKL, to activate NF- $\kappa$ B and cyclin D1 expression in the mouse mammary gland (8). In other studies we showed that in the absence of high levels of  $\beta$ -catenin IKK $\alpha$  but not IKK $\beta$  actually increased cyclin D1 expression (2). These data, taken together with a kinase-independent role of IKK $\alpha$ , in limb and skin development, illustrate the complexity and context-dependence of cytokine/IKK pathways (23).

**TNF $\alpha$ /IKK-mediated phosphorylation of  $\beta$ -catenin affects its signaling activity but does not result in a change of total  $\beta$ -catenin levels:**

In SW480 cells neither TNF $\alpha$  nor constitutively active IKKs affect total  $\beta$ -catenin protein levels or localization even though they rapidly alter  $\beta$ -catenin N-terminal phosphorylation and signaling activity. This is in contrast to the effects of APC, axin or activated GSK-3, which all result in the degradation of total  $\beta$ -catenin, concomitant with increased N-terminal phosphorylation and decreased signaling activity. These data raise the possibility that phosphorylation of the same N-terminal residues targets  $\beta$ -catenin for degradation in the context of APC/axin/GSK-3 but not in the context of TNF $\alpha$ /IKK. Although this seems contradictory it is clear that phosphorylation of  $\beta$ -catenin on serines 33, 37, 45 and threonine 41 can regulate its cellular localization and transcriptional activation functions, in addition to regulating protein degradation (17,51,59,60). In Xenopus oocytes  $\beta$ -catenin N-terminal phosphorylation regulates its transactivation properties completely independently of protein degradation (17). In addition, IKKs that have translocated to the nucleus following cytokine treatment directly affect the transcriptional activity of NF $\kappa$ B as well as targeting I $\kappa$ B for degradation (46,64). It is also possible that TNF $\alpha$ /IKK targets a small pool of transcriptionally active  $\beta$ -catenin for degradation.

Exogenous expression of E-cadherin can inhibit  $\beta$ -catenin/TCF signaling in SW480 cells in the absence of discernible changes in cytoplasmic or nuclear  $\beta$ -catenin (16). These data led the authors to conclude that most of the  $\beta$ -catenin in SW480 cells is refractory to cadherin and TCF binding and that a small pool of transcriptionally active  $\beta$ -catenin can

be modulated by E-cadherin. In other experiments we show that mechanical strain also affects  $\beta$ -catenin signaling activity and nuclear "activated"  $\beta$ -catenin without altering total  $\beta$ -catenin protein levels (Avvisato, Byers et al submitted for publication).

Our data show that  $\beta$ -catenin phosphorylated on residues 45 and 41 appears in the nucleus as early as 5 minutes following TNF $\alpha$  treatment (Fig. 6). Although no clear change in the staining of activated de-phosphorylated  $\beta$ -catenin occurs at these early time points, the antibody that recognizes activated  $\beta$ -catenin is actually directed at  $\beta$ -catenin that is dephosphorylated on residues 37 and 41 and will still recognize  $\beta$ -catenin phosphorylated on residue 45. A clear loss of activated  $\beta$ -catenin staining from the nucleus is detected 16 hours after TNF $\alpha$  treatment. This means that this pool of  $\beta$ -catenin is either phosphorylated on residues 37 and 41 or that it has been degraded and no longer exists. At this time no nuclear staining with either phospho- $\beta$ -catenin antibody is observed indicating that the latter is more likely. Consequently, one plausible interpretation of our data is that phosphorylation of a small pool of nuclear  $\beta$ -catenin by activated IKK and perhaps other kinases results in its degradation and/or alters its co-activator properties.  $\beta$ -catenin degradation could take place in the nucleus or following export into the cytoplasm. Consistent with this interpretation, one study has shown that  $\beta$ -catenin associated with the nuclear matrix in melanoma cells is a target for proteosomal degradation independently of APC (5).

### **Concluding Remarks:**

Phosphorylation of serine and threonine residues in the  $\beta$ -catenin N-terminus can both target  $\beta$ -catenin for ubiquitination and alter its co-activator properties (41,51). Several kinases are known to phosphorylate these residues. These include GSK-3 $\beta$ , casein kinase 2, IKK $\alpha$  and IKK $\beta$  and cdk2 (45). It is generally believed that, in the absence of a wnt signal, cytoplasmic  $\beta$ -catenin is constitutively phosphorylated, ubiquitinated and targeted for proteosomal degradation. Although the canonical wnt pathway is a major regulator of  $\beta$ -catenin/TCF signaling activity other factors and pathways also play an important role. For example, we showed several years ago that levels of cytoplasmic  $\beta$ -catenin and  $\beta$ -catenin/TCF signaling activity varied dramatically during the cell cycle (42). Recently it was found that cdk2/cyclin A/E phosphorylation of  $\beta$ -catenin residues 33,37,41 and 45 is responsible for its rapid degradation during the G1 phase of the cell cycle (45). It is also clear that change in  $\beta$ -catenin protein stability is not the only means whereby the cell can regulate  $\beta$ -catenin activity. For example several members of the steroid receptor superfamily, TGF $\beta$ /smad signaling, E-cadherin and mechanical force all modulate  $\beta$ -catenin/TCF signaling independently of protein degradation ((13,32,44,57) Avvisato et al submitted). TNF $\alpha$  and perhaps other IKK-activating cytokines can now be added to this list of  $\beta$ -catenin/TCF regulators.

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**Figure 1. TNF $\alpha$  and IKK affect  $\beta$ -catenin signaling independently of APC**

**A:** SW480 cells were transfected with 0.1ug of TOPFLASH or NF- $\kappa$ B reporter. After 24 hrs, cells were treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs and assayed for the reporter activity. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

**B:** 293 cells were transfected with 0.1ug of TOPFLASH or NF- $\kappa$ B reporter. After 24 hrs, cells were treated with 20ng/ml of TNF $\alpha$  for 16 hrs and assayed for reporter activity. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

**C:** SW480 cells were transfected with 0.15 ug of a siamois reporter, or a siamois mutant without a TCF binding site. After 24 hrs, cells were treated with 20ng/ml of TNF $\alpha$  for 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted.

**D:** SW480 cells were transfected with 0.1 ug of NF- $\kappa$ B inducing kinase (NIK) together with 0.1ug of TOPFLASH or NF- $\kappa$ B for 24 hrs. Luciferase activities were normalized with renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

**E:** 1.0 ug of constitutively active IKK mutants (CA IKK $\alpha$  or CA IKK $\beta$ ) were transfected into SW480 cells together with 0.1ug of TOPFLASH or NF- $\kappa$ B reporter. 24 hours later, some cells were treated with 20ng/ml of TNF $\alpha$  for 16 hrs.

**F:** SW480 cells

were transfected with 1.0ug of kinase dead IKK mutants for 24 hrs. **G:** SW480 cells transfected with 0.1ug of constitutively active IKK mutants (CA IKK) and 0.1 ug of cyclin D1 (-163) reporter or 0.1 ug of cyclin D1 (-163) reporter with a mutated TCF/LEF binding site. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted.

**Figure 2: The IKK complex mediates the effects of TNF $\alpha$  but not APC on  $\beta$ -catenin signaling:**

**A:** SW480 cells were transfected with 0.1ug of TOPFLASH or NF- $\kappa$ B reporters and increasing amounts of dominant negative IKK mutants (DN IKK $\alpha$  and DN IKK $\beta$ ) (0.05-1.0ug) for 24 hrs. After which, cells were treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. **B:** SW480 cells were transfected with 0.2ug of APC, 0.1ug of TOPFLASH or NF- $\kappa$ B reporter and increasing amounts of DN IKK mutants (0.2-1.0ug) for 24 hrs, after which, cells were treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. **C:** Wild Type MEF cells were treated with 25nM of IKK $\alpha$  siRNA for 72 hours. Media was changed and 0.1ug of TOPFLASH or NF- $\kappa$ B reporter was transfected for 24 hrs and cells treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \*

denotes statistical significance where  $p < 0.05$ . **D:** Wild Type MEF cells were treated with 100nM of IKK $\beta$  siRNA for 72 hours. Media was changed and 0.1ug of TOPFLASH or NF- $\kappa$ B reporter was transfected for 24 hrs and treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ . **E:** Wild type MEFs (hatched), IKK $\alpha$  (-/-) (dotted), IKK $\beta$  (-/-) (squares) were transfected with 0.1ug of TOPFLASH or NF- $\kappa$ B reporter for 24 hrs and treated with 20ng/ml of TNF $\alpha$  for a further 16 hrs. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. **F:** SW480 cells were transfected with 0.6ug of each of the following dominant negative mutants, NIK, PI3 kinase, AKT, TAK for 24 hrs. GSK-3 activity was blocked by incubation of cells in 20 mM of Li $^+$  for 24 h.

**Figure 3: TNF $\alpha$  repression of  $\beta$ -catenin activity is independent of NF $\kappa$ B signaling activity:**

**A:** Wild type mouse embryonic fibroblast cells were treated with 80nM of p65 siRNA or non-specific siRNA, (NSRNAi) for 72 hrs Western analysis of wild type MEFs treated with 80nM of p65 RNA show significant reduction of p65 protein compared to NSRNAi and mock transfections. Bottom panel indicates  $\alpha$ -tubulin loading control. **B:** p65 siRNA treated cells were transfected with 0.1ug of TOPFLASH reporter and treated with TNF for 16h. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where  $p < 0.05$ .

C: p65 siRNA treated cells were transfected with 0.1ug of NF $\kappa$ B reporter and treated with TNF for 16h. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. \* denotes statistical significance where p< 0.05.

**Figure 4: TNF $\alpha$  treatment does not decrease total  $\beta$ -catenin levels in SW480 and HEK293 cells:**

**A:** Top panel: SW480 cells were treated with 20ng/ml TNF $\alpha$  harvested after 16 hrs and the cytoplasmic fraction used to run a western analysis as described in Materials and Methods. Cells were probed with a  $\beta$ -catenin antibody that recognizes the C-terminal. Lower panel: Loading control  $\alpha$ -tubulin. **B:** As described in A using 293 cells.

**Figure 5: Activated IKKs regulate the localization of activated, de-phosphorylated  $\beta$ -catenin:** SW480 cells were transfected with 1.0ug of CA IKK $\alpha$  or CA IKK $\beta$  mutants for 24 hrs and cells were harvested and stained as described in Materials and Methods. Results for CAIKK $\alpha$  and CAIKK $\beta$  were identical and only IKK $\beta$  is shown here (40X):

**A:** SW480 cells were stained for flag tagged CAIKK $\beta$  showed strong cytoplasmic staining with little nuclear staining. **B:** SW480 cells stained for  $\beta$ -catenin C-terminal (Texas red) showed predominantly nuclear with some cytoplasmic staining. **C:** Merged image of panel A and panel B.

**D:** SW480 cells were stained using an antibody specific for  $\beta$ -catenin de-phosphorylated on serine 37 and threonine 41 (green). **E:** SW480 cells were stained for transfected CA

IKK $\beta$  (Red). **F:** Combined image of D) and E). SW480 cells transfected with CA IKK mutants had little or no nuclear staining (see arrow) when probed with an antibody specific for the de-phosphorylated form of  $\beta$ -catenin. **Figure G-J:** Analysis of the number of cells with and without nuclear de-phosphorylated  $\beta$ -catenin after transfection with 1.0ug of CA IKK $\alpha$  or CA IKK $\beta$ . 10 fields were viewed and the number of cells with nuclear activated  $\beta$ -catenin counted and the average calculated. There is a significant decrease in number of cells with nuclear staining after transfection with CA IKK mutants.

**Figure 6: TNF $\alpha$  decreases nuclear dephosphorylated  $\beta$ -catenin:**

**A:** Control SW480 cells. **B:** SW480 cells were treated with 20ng/ml of TNF $\alpha$  for 16 hrs and stained for  $\beta$ -catenin de-phosphorylated on residues 37 and 41. At similar exposure times, treatment with TNF $\alpha$  resulted in absent or much less intense nuclear staining when compared to control cells. **C-D:** SW480 cells stained with polyclonal antibody that recognizes  $\beta$ -catenin phosphorylated at residues Thr41/Ser45, before (C) and after treatment with TNF $\alpha$  for 30 minutes (D). **E-F:** SW480 stained with polyclonal antibody that recognizes  $\beta$ -catenin phosphorylated at residues S33, S37, T41 before (E) and after (F) treatment with TNF $\alpha$  for 30 min. **G:** Quantitation of nuclear staining of dephosphorylated  $\beta$ -catenin before and after TNF $\alpha$  treatment.

**Figure 7: TNF $\alpha$  trans-repression of  $\beta$ -catenin activity involves phosphorylation of N-terminal serine and threonine residues:**

**A:** Parental MEF cells were transfected with 0.5ug of wild type  $\beta$ -catenin (WT), S45A mutant or S33,37,41,45 mutant and 0.1 ug of TOPFLASH reporter for 24 hrs and treated with 20ng/ml TNF $\alpha$  for a further 16 hrs. Mutant  $\beta$ -catenin was insensitive to the effects of TNF $\alpha$ . Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. **B-C:** As A but using IKK  $\alpha$  (-/-) (B) and IKK $\beta$  (-/-) (C) cells. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test. **D:** Parental HCT 116 cell line (WT $^+$ /Mut $^+$ ) in which a somatic mutation has deleted serine 45 in one  $\beta$ -catenin allele, HCT-116 cells in which either the wild-type or mutant allele was removed by somatic cell knockout were transfected with 0.1ug of TOPFLASH reporter for 24 hrs and treated with TNF $\alpha$  for a further 16h. Luciferase activities were normalized to renilla and plotted as % control. The average of three experiments, +/- standard deviation is plotted. Statistical analysis was performed using paired Student's T-test.

**Figure 8:** Both wildtype (WT) (A) and IKK $\alpha$ -/- (B) embryos were stained with mouse IgG and hematoxylin. No background staining was observed with the IgG control antibody. Note the thickened epidermis of the IKK $\alpha$ -/- embryo. Because hematoxylin masked the nuclear  $\beta$ -catenin staining, the embryos were stained with  $\beta$ -catenin alone.

Membrane staining was observed in both WT (C) and IKK $\alpha$ -/- skin (D). However, an increased number of epidermal cells with nuclear  $\beta$ -catenin (arrows) was observed in the IKK $\alpha$ -/- mouse. Cyclin D1 expression was also increased in the basal aspects of the epidermis of the IKK $\alpha$ -/- mouse. (E-H).

#### Reference List

1. **Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler.** 1997.  $\beta$ -catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**:3797-3804.
2. **Albanese, C., K. Wu, M. D'Amico, C. Jarrett, D. Joyce, J. Hughes, J. Hulit, T. Sakamaki, M. Fu, A. Ben Ze'ev, J. F. Bromberg, C. Lamberti, U. Verma, R. B. Gaynor, S. W. Byers, and R. G. Pestell.** 2003. IKKalpha Regulates Mitogenic Signaling through Transcriptional Induction of Cyclin D1 via Tcf. *Mol.Biol Cell* **14**:585-599.
3. **Amit, S. and Y. Ben Neriah.** 2003. NF-kappaB activation in cancer: a challenge for ubiquitination- and proteasome-based therapeutic approach. *Semin.Cancer Biol* **13**:15-28.
4. **Amit, S., A. Hatzubai, Y. Birman, J. S. Andersen, E. Ben Shushan, M. Mann, Y. Ben Neriah, and I. Alkalay.** 2002. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev.* **16**:1066-1076.
5. **Bonvini, P., S. G. Hwang, M. El Gamil, P. Robbins, J. S. Kim, J. Trepel, and L. Neckers.** 2000. Nuclear beta-catenin displays GSK-3beta- and APC-independent proteasome sensitivity in melanoma cells. *Biochim.Biophys.Acta* **1495**:308-318.
6. **Brannon, M., M. Gomperts, L. Sumoy, R. T. Moon, and D. Kimelman.** A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev.* **11**: 2359-2370. 1997.
7. **Burow, M. E., C. B. Weldon, L. I. Melnik, B. N. Duong, B. M. Collins-Burow, B. S. Beckman, and J. A. McLachlan.** 2000. PI3-K/AKT regulation of NF-kappaB signaling events in suppression of TNF-induced apoptosis. *Biochem.Biophys.Res Commun.* **271**:342-345.

8. **Cao, Y., G. Bonizzi, T. N. Seagroves, F. R. Greten, R. Johnson, E. V. Schmidt, and M. Karin.** 2001. IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell* **107**:763-775.
9. **Chen, S. Q., D. C. Guttridge, Z. B. You, Z. C. Zhang, A. Fribley, M. W. Mayo, J. Kitajewski, and C. Y. Wang.** 2001. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. *J. Cell Biol* **152**:87-96.
10. **Delhase, M., M. Hayakawa, Y. Chen, and M. Karin.** 1999. Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation [see comments]. *Science* **284**:309-313.
11. **Deng, J., S. A. Miller, H. Y. Wang, W. Xia, Y. Wen, B. P. Zhou, Y. Li, S. Y. Lin, and M. C. Hung.** 2002. beta-catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. *Cancer Cell* **2**:323-334.
12. **Devin, A., Y. Lin, S. Yamaoka, Z. Li, M. Karin, and Z. Liu.** 2001. The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. *Mol. Cell Biol* **21**:3986-3994.
13. **Easwaran, V., M. Pishvaian, Salimuddin, and S. Byers.** 1999. Cross regulation of beta catenin/LEF/TCF and retinoid signaling pathways. *Current Biology* **9**:1415-1418.
14. **Ghosh, S. and M. Karin.** 2002. Missing pieces in the NF-kappaB puzzle. *Cell* **109 Suppl**:S81-S96.
15. **Ghosh, S., M. J. May, and E. B. Kopp.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev Immunol.* **16**:225-260.
16. **Gottardi, C. J., E. Wong, and B. M. Gumbiner.** 2001. E-cadherin suppresses cellular transformation by inhibiting beta- catenin signaling in an adhesion-independent manner. *J. Cell Biol.* **153**:1049-1060.
17. **Guger, K. A. and B. M. Gumbiner.** 2000. A mode of regulation of beta-catenin signaling activity in Xenopus embryos independent of its levels. *Dev. Biol* **223**:441-448.
18. **Henderson, B. R.** 2000. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat. Cell Biol* **2**:653-660.
19. **Hewitt, R. E., A. McMarlin, D. Kleiner, R. Wersto, P. Martin, M. Tsokos, G. W. Stamp, W. G. Stetler-Stevenson, and M. Tsoskas.** 2000. Validation of a model of colon cancer progression. *J Pathol.* **192**:446-454.

20. **Hinoi, T., H. Yamamoto, M. Kishida, S. Takada, S. Kishida, and A. Kikuchi.** 2000. Complex formation of adenomatous polyposis coli gene product and axin facilitates glycogen synthase kinase-3 beta-dependent phosphorylation of beta-catenin and down-regulates beta-catenin. *J Biol Chem* **275**:34399-34406.
21. **Hoeflich, K. P., J. Luo, E. A. Rubie, M. S. Tsao, O. Jin, and J. R. Woodgett** 2000. Requirement for glycogen synthase kinase-3 beta in cell survival and NF-kappaB activation. *Nature*. **406**:86-90.
22. **Hu, Y., V. Baud, M. Delhase, P. Zhang, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284**:316-320.
23. **Hu, Y., V. Baud, T. Oga, K. I. Kim, K. Yoshida, and M. Karin.** 2001. IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature* **410**:710-714.
24. **Johns, L. D., T. Sarr, and G. E. Ranges.** 1994. Inhibition of ceramide pathway does not affect ability of TNF-alpha to activate nuclear factor-kappa B. *J Immunol.* **152**:5877-5882.
25. **Karin, M.** 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. *Oncogene* **18**:6867-6874.
26. **Kawahara, K., T. Morishita, T. Nakamura, F. Hamada, K. Toyoshima, and T. Akiyama.** 2000. Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin. *J Biol Chem* **275**:8369-8374.
27. **Kim, J. S., H. Crooks, A. Foxworth, and T. Waldman.** 2002. Proof-of-principle: oncogenic beta-catenin is a valid molecular target for the development of pharmacological inhibitors. *Mol. Cancer Ther.* **1**:1355-1359.
28. **Kitagawa, M., S. Hatakeyama, M. Shirane, M. Matsumoto, N. Ishida, K. Hattori, I. Nakamichi, A. Kikuchi, and K. Nakayama.** 1999. An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. *EMBO J.* **18**:2401-2410.
29. **Lamberti, C., K. M. Lin, Y. Yamamoto, U. Verma, I. M. Verma, S. Byers, and R. B. Gaynor.** 2001. Regulation of beta-catenin function by the IkappaB kinases. *J Biol Chem* **276**:42276-42286.
30. **Landesman-Bollag, E., R. Romieu-Mourez, D. H. Song, G. E. Sonenshein, R. D. Cardiff, and D. C. Seldin.** 2001. Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**:3247-3257.

31. **Landesman-Bollag, E., D. H. Song, R. Romieu-Mourez, D. J. Sussman, R. D. Cardiff, G. E. Sonenshein, and D. C. Seldin.** 2001. Protein kinase CK2: signaling and tumorigenesis in the mammary gland. *Mol. Cell Biochem.* **227**:153-165.
32. **Letamendia, A., E. Labbe, and L. Attisano.** 2001. Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. *J Bone Joint Surg Am* **83-A Suppl 1**:S31-S39.
33. **Li, Q., Q. Lu, J. Y. Hwang, D. Buscher, K. F. Lee, J. C. Izpisua-Belmonte, and I. M. Verma.** 1999. IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev.* **13**:1322-1328.
34. **Li, Y., J. Kang, J. Friedman, L. Tarassishin, J. Ye, A. Kovalenko, D. Wallach, and M. S. Horwitz.** 1999. Identification of a cell protein (FIP-3) as a modulator of NF-kappaB activity and as a target of an adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **96**:1042-1047.
35. **Li, Z. W., W. Chu, Y. Hu, M. Delhase, T. Deerinck, M. Ellisman, R. Johnson, and M. Karin.** 1999. The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J. Exp. Med.* **189**:1839-1845.
36. **Lin, S. Y., W. Xia, J. C. Wang, K. Y. Kwong, B. Spohn, Y. Wen, R. G. Pestell, and M. C. Hung.** 2000. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U.S.A.* **97**:4262-4266.
37. **Masui, O., Y. Ueda, A. Tsumura, M. Koyanagi, M. Hijikata, and K. Shimotohno.** 2002. RelA suppresses the Wnt/beta-catenin pathway without exerting trans-acting transcriptional ability. *Int J Mol. Med.* **9**:489-493.
38. **Mercurio, F., B. W. Murray, A. Shevchenko, B. L. Bennett, D. B. Young, J. W. Li, G. Pascual, A. Motiwala, H. Zhu, M. Mann, and A. M. Manning.** 1999. IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Mol. Cell Biol.* **19**:1526-1538.
39. **Mercurio, F., H. Zhu, B. W. Murray, A. Shevchenko, B. L. Bennett, J. Li, D. B. Young, M. Barbosa, M. Mann, A. Manning, and A. Rao.** 1997. IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF- kappaB activation. *Science* **278**:860-866.
40. **Nakamura, T., F. Hamada, T. Ishidate, K. Anai, K. Kawahara, K. Toyoshima, and T. Akiyama.** 1998. Axin, an inhibitor of the Wnt signalling pathway, interacts with beta- catenin, GSK-3beta and APC and reduces the beta- catenin level. *Genes Cells* **3**:395-403.

41. **Orford, K., C. Crockett, J. P. Jensen, A. M. Weissman, and S. W. Byers.** 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta catenin. *JBC* **272**:24735-24738.
42. **Orford, K., C. C. Orford, and S. W. Byers.** 1999. Exogenous expression of beta catenin regulates contact inhibition, anchorage-independent growth, anoikis and radiation-induced cell-cycle arrest. *J. Cell Biol* **146**:1-13.
43. **Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner.** 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**:82-85.
44. **Palmer, H. G., J. M. Gonzalez-Sancho, J. Espada, M. T. Berciano, I. Puig, J. Baulida, M. Quintanilla, A. Cano, H. A. de, M. Lafarga, and A. Munoz.** 2001 Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol.* **154**:369-387.
45. **Park, C. S., S. I. Kim, M. S. Lee, C. Y. Youn, D. J. Kim, E. H. Jho, and W. K. Song.** 2004. Modulation of beta-catenin phosphorylation/degradation by cyclin-dependent kinase 2. *J Biol Chem* **279**:19592-19599.
46. **Prajapati, S. and R. B. Gaynor.** 2002. Regulation of Ikappa B kinase (IKK)gamma /NEMO function by IKKbeta -mediated phosphorylation. *J Biol Chem* **277**:24331-24339.
47. **Provost, E., Y. Yamamoto, I. Lizardi, J. Stern, T. G. D'Aquila, R. B. Gaynor, and D. L. Rimm.** 2003. Functional correlates of mutations in beta-catenin exon 3 phosphorylation sites. *J Biol Chem* **278**:31781-31789.
48. **Ray, R., R. Cabal-Manzano, A. R. Moser, T. Waldman, L. M. Zipper, A. Aigner, S. W. Byers, A. T. Riegel, and A. Wellstein.** 2003. Up-regulation of fibroblast growth factor-binding protein, by beta-catenin during colon carcinogenesis. *Cancer Res* **63**:8085-8089.
49. **Rothwarf, D. M., E. Zandi, G. Natoli, and M. Karin.** 1998. IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* **395**:297-300.
50. **Russo, M. P., B. L. Bennett, A. M. Manning, D. A. Brenner, and C. Jobin.** 2002. Differential requirement for NF-kappaB-inducing kinase in the induction of NF-kappaB by IL-1beta, TNF-alpha, and Fas. *Am J Physiol Cell Physiol* **283**:C347-C357.
51. **Sadot, E., M. Conacci-Sorrell, J. Zhurinsky, D. Shnizer, Z. Lando, D. Zharhary, Z. Kam, A. Ben Ze'ev, and B. Geiger.** 2002. Regulation of S33/S37 phosphorylated beta-catenin in normal and transformed cells. *J Cell Sci* **115**:2771-2780.

52. **Sakanaka, C.** 2002. Phosphorylation and regulation of beta-catenin by casein kinase I epsilon. *J Biochem.(Tokyo)* **132**:697-703.
53. **Schwabe, R. F. and D. A. Brenner.** 2002. Role of glycogen synthase kinase-3 in TNF-alpha-induced NF-kappaB activation and apoptosis in hepatocytes. *Am J Physiol Gastrointest.Liver Physiol* **283**:G204-G211.
54. **Shtutman, M., J. Zhurinsky, I. Simcha, C. Albanese, M. D'Amico, R. Pestell, and Z. Ben.** 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc.Natl.Acad.Sci.U.S.A.* **96**:5522-5527.
55. **Staal, F. J., M. M. Noort, G. J. Strous, and H. C. Clevers.** 2002. Wnt signals are transmitted through N-terminally dephosphorylated beta-catenin. *EMBO Rep.* **3**:63-68.
56. **Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira.** 1999. Limb and skin abnormalities in mice lacking IKKalpha. *Science* **284**:313-316.
57. **Truica, C. I., S. Byers, and E. P. Gelmann.** 2000. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Res* **60**:4709-4713.
58. **van de Wetering, M., R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Louriero, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Martini, and H. Clevers.** 1997. Armadillo co-activates transcription driven by the product of the drosophila segment polarity gene dTCF. *Cell* **88**:789-799.
59. **van Noort, M., J. Meeldijk, Z. R. van der, O. Destree, and H. Clevers.** 2002. Wnt signaling controls the phosphorylation status of beta-catenin. *J Biol Chem* **277**:17901-17905.
60. **van Noort, M., W. M. van de, and H. Clevers.** 2002. Identification of two novel regulated serines in the N terminus of beta-catenin. *Exp.Cell Res* **276**:264-272.
61. **Vermeulen, L., G. De Wilde, S. Notebaert, W. Vanden Berghe, and G. Haegeman.** 2002. Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. *Biochem.Pharmacol.* **64**:963-970.
62. **Webster, G. A. and N. D. Perkins.** 1999. Transcriptional cross talk between NF-kappaB and p53. *Mol.Cell Biol* **19**:3485-3495.
63. **Woronicz, J. D., X. Gao, Z. Cao, M. Rothe, and D. V. Goeddel.** 1997. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science* **278**:866-869.
64. **Yamamoto, Y., D. W. Kim, Y. T. Kwak, S. Prajapati, U. Verma, and R. B. Gaynor.** 2001. IKKgamma /NEMO facilitates the recruitment of the IkappaB proteins into the IkappaB kinase complex. *J Biol Chem* **276**:36327-36336.

65. **Yamaoka, S., G. Courtois, C. Bessia, S. T. Whiteside, R. Weil, F. Agou, H. E. Kirk, R. J. Kay, and A. Israel.** 1998. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**:1231-1240.
66. **Zandi, E. and M. Karin.** 1999. Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. *Mol. Cell Biol* **19**:4547-4551.
67. **Zandi, E., D. M. Rothwarf, M. Delhase, M. Hayakawa, and M. Karin.** 1997. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91**:243-252.
68. **Zhu, A. J. and F. M. Watt.** 1999. beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development* **126**:2285-2298.

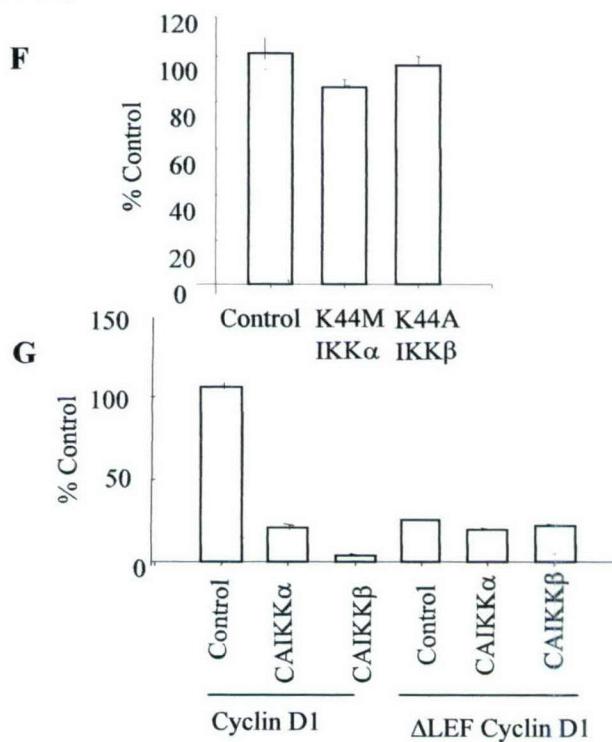
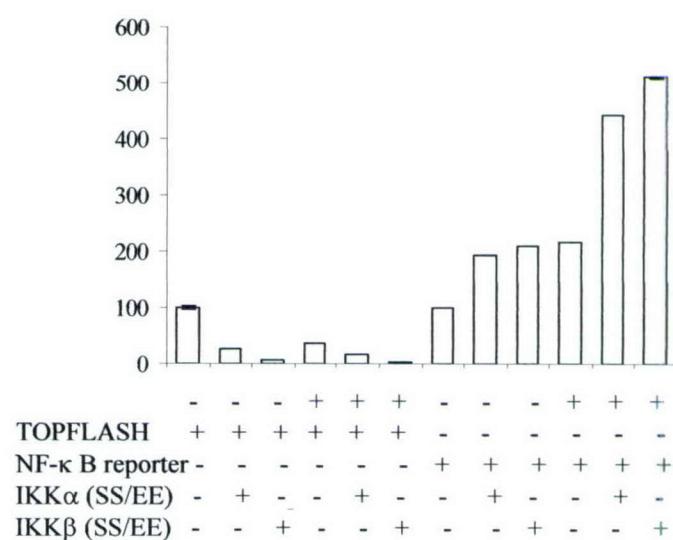
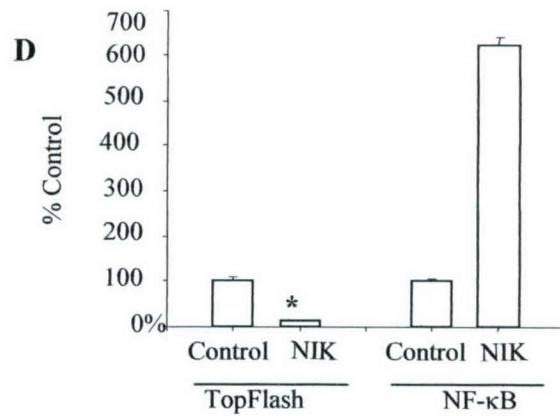
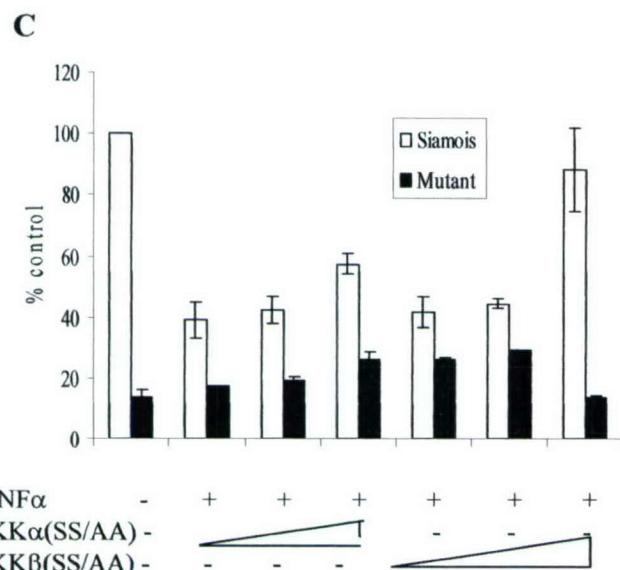
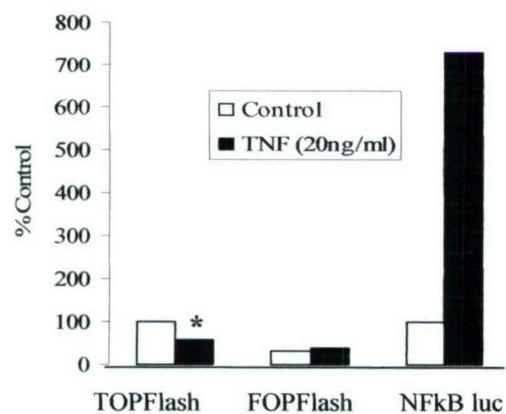
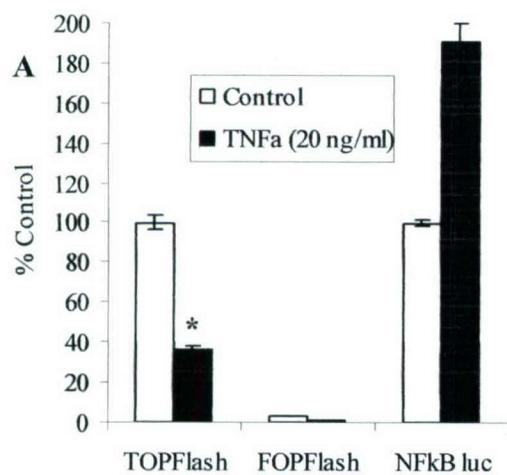
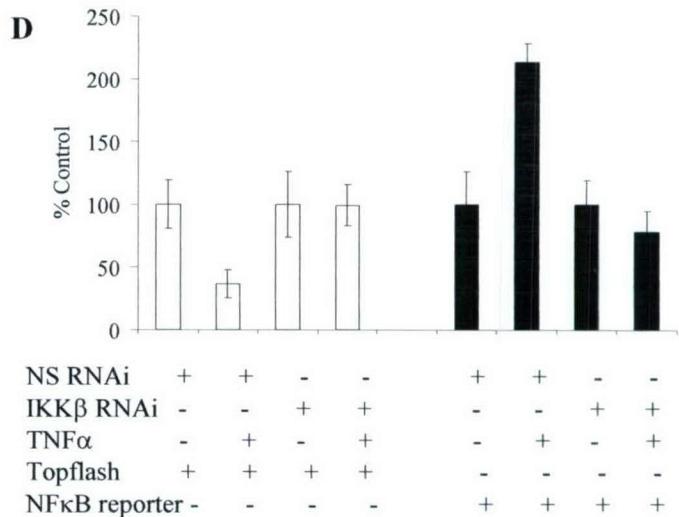
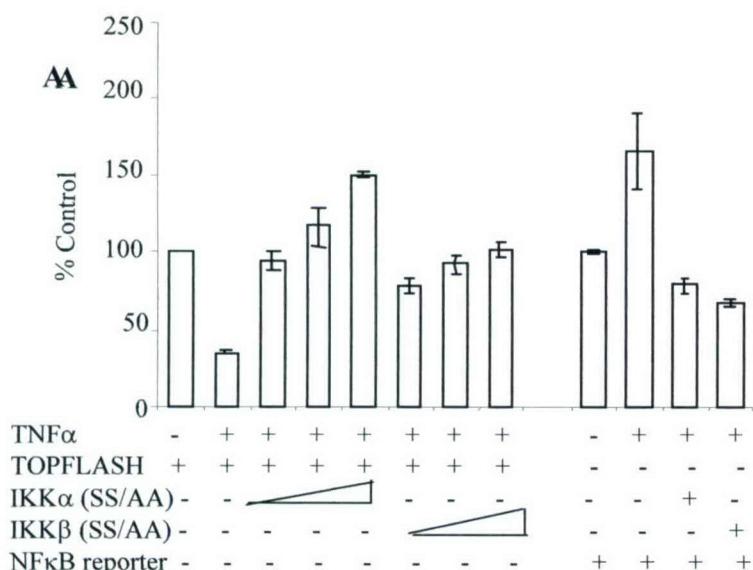


Figure 1



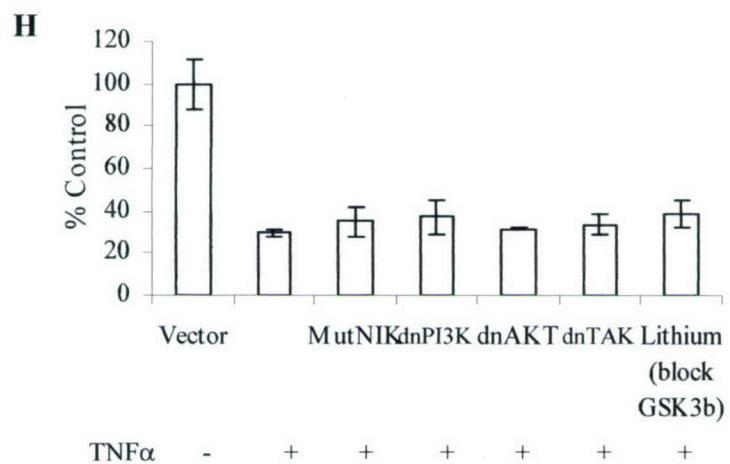


Figure 2

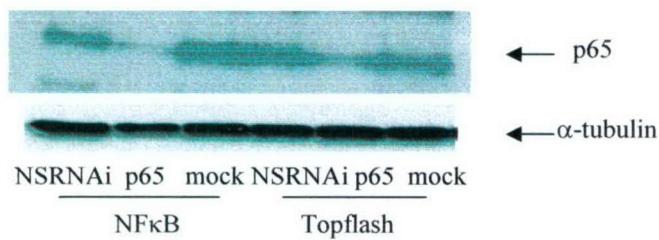
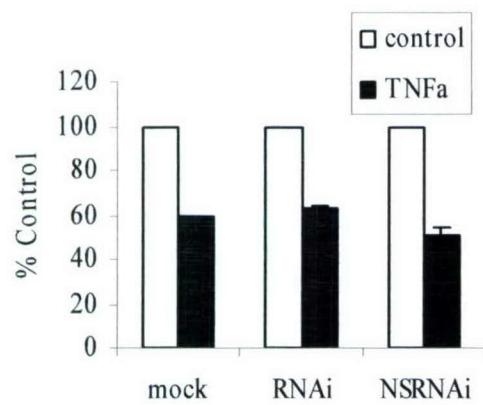
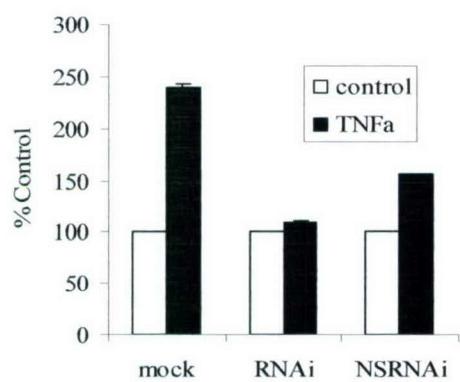
**A****B****C**

Figure 3

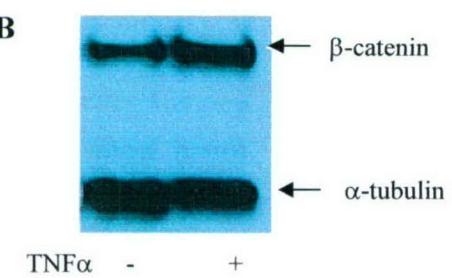
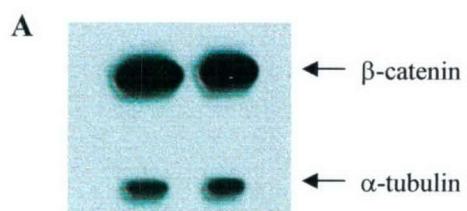
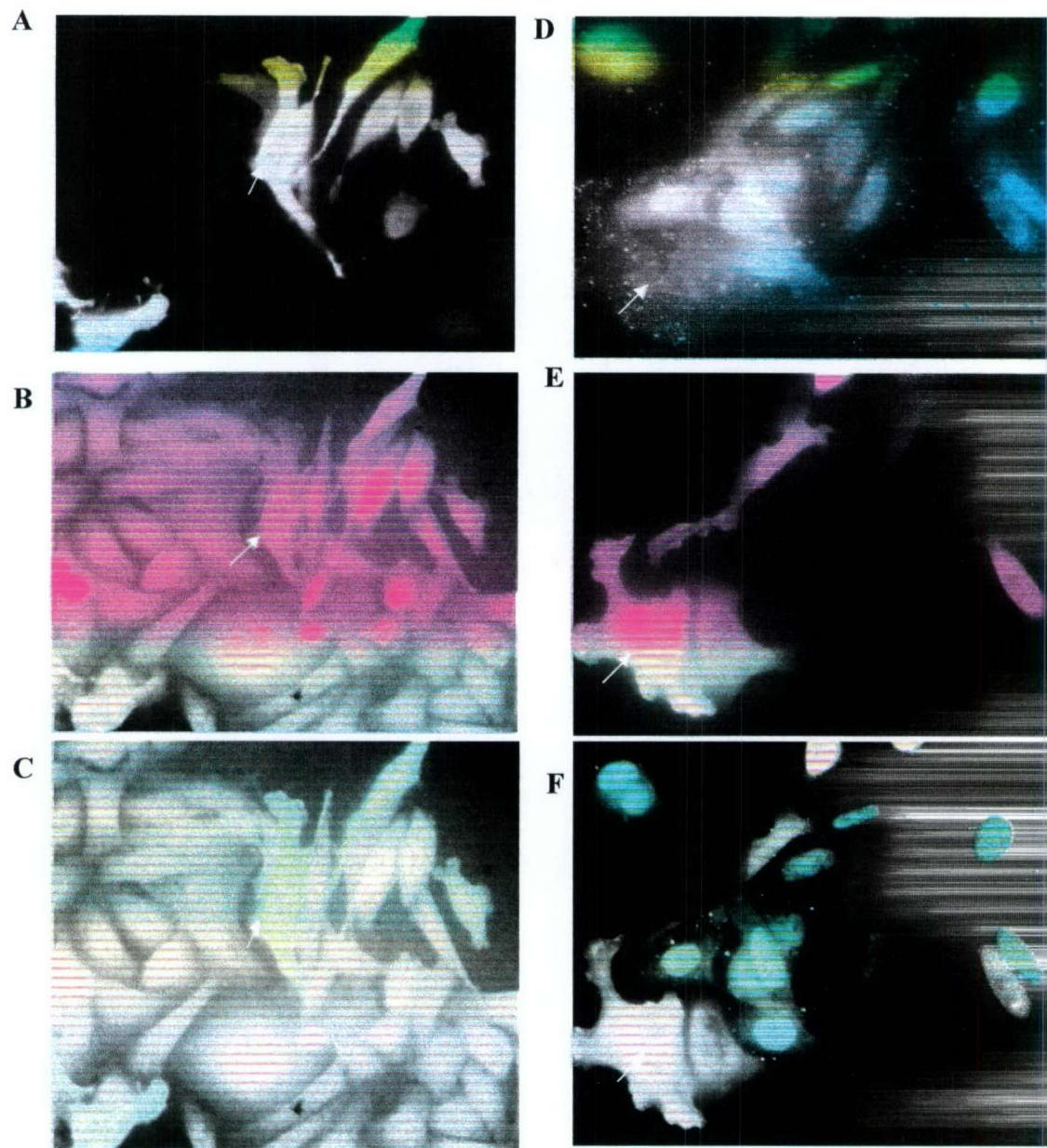
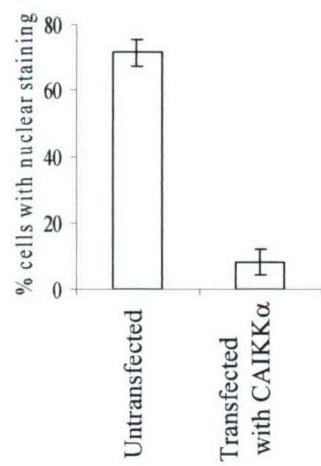


Figure 4



**G**



**H**

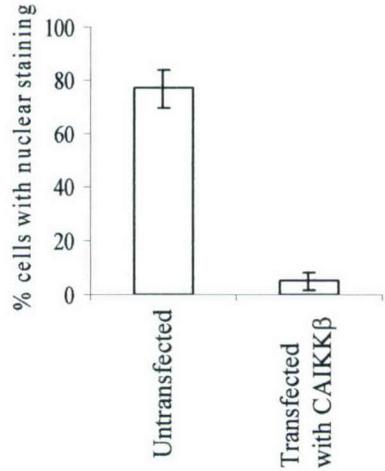


Figure 5

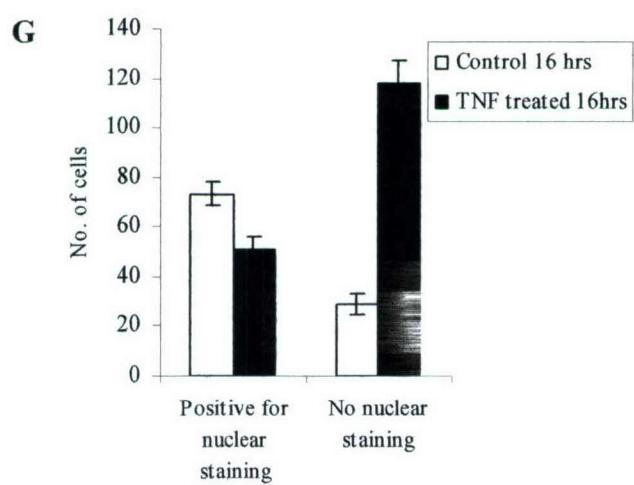
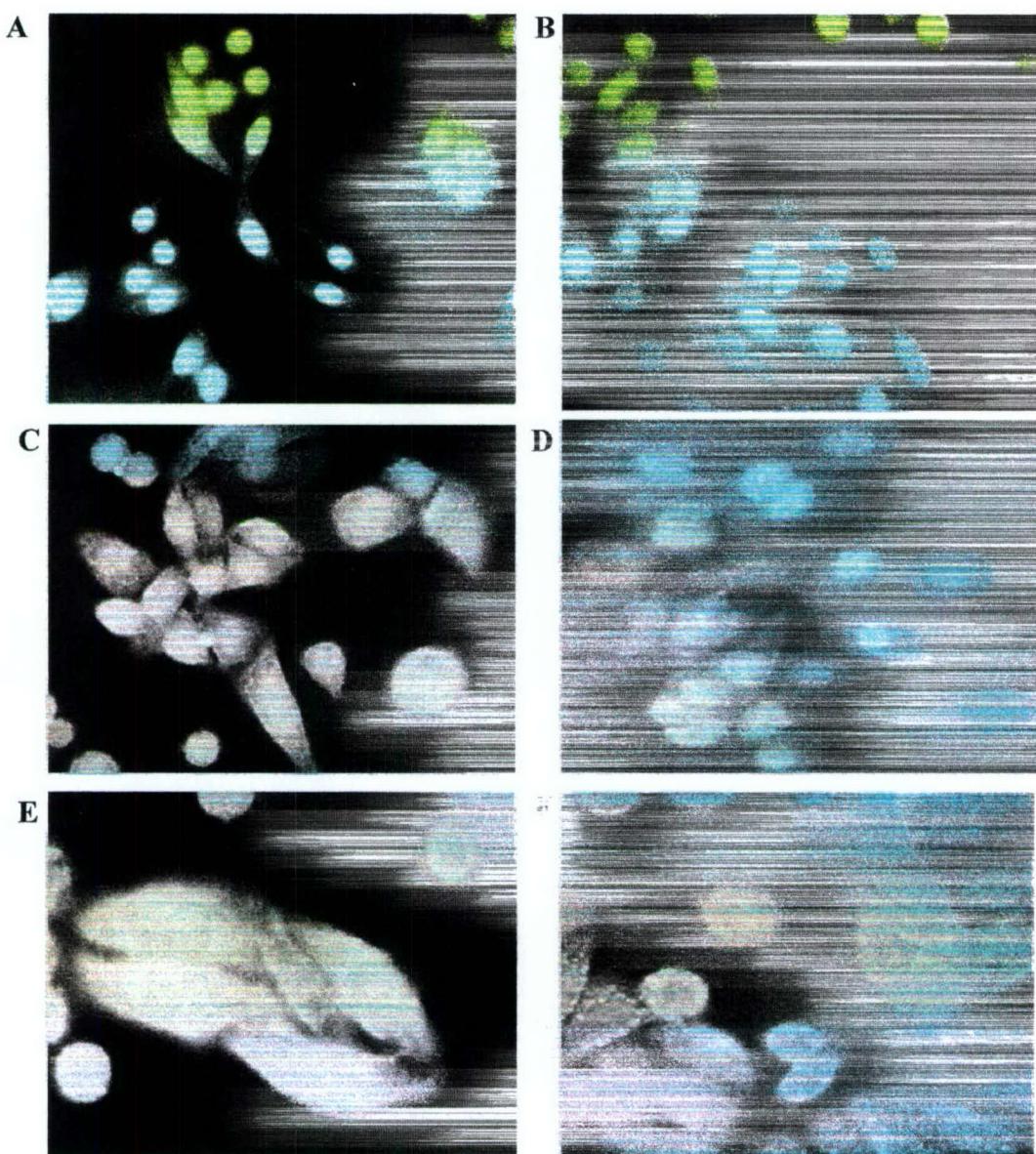


Figure 6

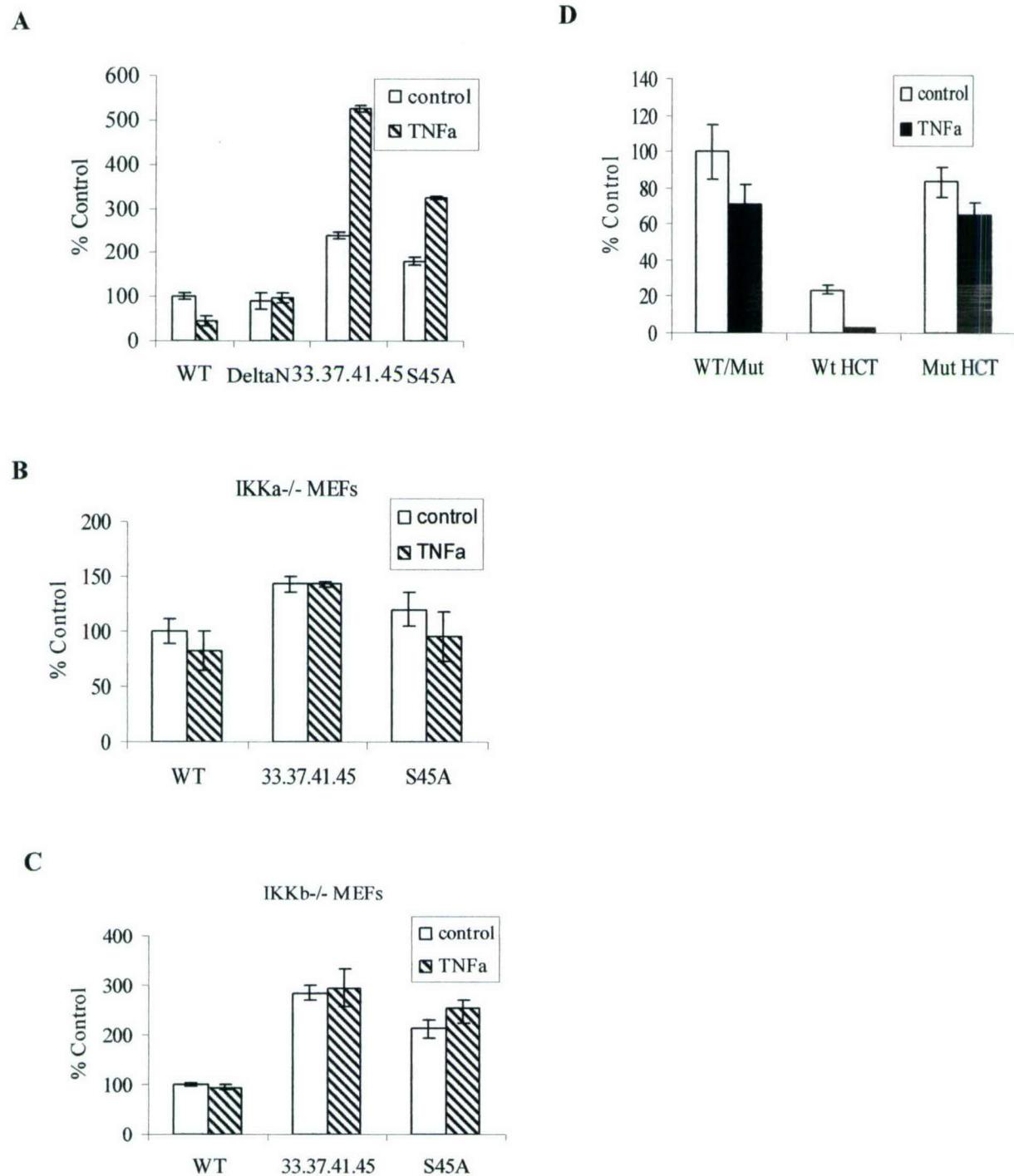


Figure 7

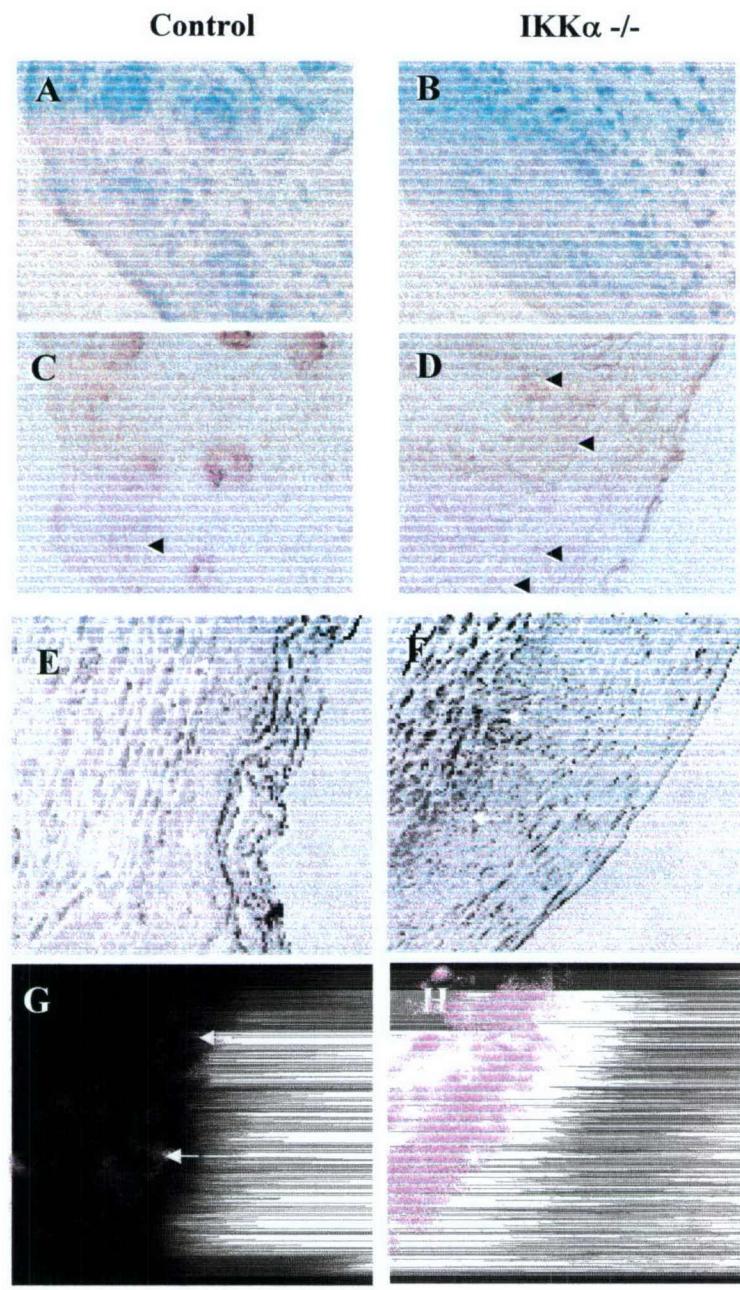


Figure 8